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VOLUME 36 JULY 1958 NUMBER 4

STUDIES ON FUSARIUM WILT OF BANANAS

III. INFLUENCE OF SOIL FUNGITOXINS ON BEHAVIOR OF F. OXYSPORUM f. CUBENSE IN SOIL EXTRACTS AND DIFFUSATES[1]

R. H. STOVER

Abstract

Aqueous extracts from soil obtained (1) by mixing equal volumes of soil and distilled water and filtering until clear, (2) by displacing the soil solution with ethyl alcohol, and (3) by soil perfusion were inhibitory to germination, hyphal growth, sporulation, and chlamydospore formation by *Fusarium oxysporum* f. *cubense*. In general, acid loam extracts were less fungitoxic than alkaline clay loam extracts and differences were not related to extract pH. Fungitoxins were reduced by filtering extracts through charcoal, by evaporating or dialyzing the extracts, and by air-drying soil before extracting. Fungitoxins were greatly reduced by filtration through sintered-glass or Seitz filters. Heat sterilization or fumigation with ethylene oxide destroyed most of the fungitoxic effect. However, differences in growth and sporulation between heat-sterilized loam and clay loam extracts and diffusates were often apparent. *Fusarium* germination, hyphal growth, sporulation, and chlamydospore formation on agar films were inhibited by fungitoxins diffusing through cellulose membranes and by addition of non-sterile soil to 1.5% water agar.

High concentrations of nutrients masked and low concentrations reduced effects of fungitoxins in soil extracts and diffusates. The addition of dextrose, glutamic acid, yeast extract, banana sap, or banana roots to extracts stimulated growth and sporulation. Yeast extract or glutamic acid stimulated sporulation at concentrations of 10 p.p.m., particularly where the bacterial population in the extract had been reduced by filtering through sintered glass.

Fungitoxins and nutrients interacting, often appeared to act independently on hyphal growth, sporulation, and chlamydospore formation, stimulating or inhibiting one but not the others. Both sporulation and chlamydospore formation, in general, were more sensitive to fungitoxins and nutrients than was hyphal growth. In addition, morphogenic responses of the yellow and non-yellow *Fusarium* clones to fungitoxins and nutrients were markedly different. Both germination and hyphal growth were greater with the yellow than the non-yellow clone in all extracts. In general, chlamydospores were produced more frequently and in greater numbers in agar films containing diffusates from acid loam than alkaline clay loam. Chlamydospores were formed most rapidly and in greatest abundance on sterile soil-amended or plain agar after 5 to 6 days. In aqueous extracts chlamydospores, if produced, formed after 3 to 5 days. Conidia, if produced, formed within 18 to 42 hours regardless of substrates.

The data indicated that fungitoxins in extracts and diffusates were associated with the bacterial soil flora since measures that reduced or altered this flora were reflected in a response by *Fusarium*.

Introduction

Previous studies (18, 19) showed that *Fusarium oxysporum* f. *cubense* (E.F.S.) Sny. and Hans., the cause of banana wilt or Panama disease, sporulated better in some soils than in others. In conjunction with these studies, experiments were undertaken with aqueous extracts and diffusates from the

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Contribution from the Department of Plant Pathology, The Vining C. Dunlap Laboratory of Tropical Research, United Fruit Company, La Lima, Honduras.

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soils to determine if the factors influencing sporulation were present in them, and to study some of the fungus-influencing properties of the soil solution. The first detailed results of these studies, a preliminary report of which has appeared (17), are presented in this paper.

Methods

For soil extract experiments, field soils of similar moisture content maintained outdoors in large boxes were used directly, or these soils were air-dried, the large lumps crumbled, mixed, and placed in 12-in. pots. In some pots the soils were kept air-dry, in others tap water was added until soil moisture measured 25, 50, or 75% of saturation. Moisture levels were maintained by adding water to replace that lost by evaporation, losses being determined by biweekly weighings. Aqueous soil extracts were prepared approximately three weeks after first moistening. With soils of varying moisture content, all samples were brought to saturation before mixing 1000 cc. of soil with 1 liter of distilled water. In soils taken directly from outdoor boxes, at moisture levels varying from 25 to 50% of saturation, 1000 cc. was mixed with 1 liter of distilled water. The suspension was shaken for about one minute and filtered through coarse and then fine filter paper until clear. In some experiments, extracts were passed through a Seitz or sintered-glass filter, or through filter paper after the addition of 0.5 g. of animal charcoal (Norit) per 100 ml. of extract. Heat sterilization was accomplished in the autoclave at 15 lbs. pressure for 10 minutes. Where pH adjustments were made, $Ca(OH)_2$, NaOH, and dilute H_2SO_4 were used. In nutritional studies, 1 ml. amounts of a 1% solution of dextrose, 0.1% solution of L-glutamic acid, banana sap, or yeast extract were added to Petri dishes containing 5 ml. of soil extract.

Displaced soil solutions were obtained by packing screened dry soil in glass columns (12 × ¾ in.) containing a stoppered glass tube outlet in the bottom. The soils were moistened with water to 25–50% saturation and, after 3 weeks' incubation, ethyl alcohol was poured into the top of the column and the displaced soil solution collected at the bottom. Soil perfusates were obtained by the Lees and Quastel method as modified by Chase (3).

Cellulose film soil bags (Fig. 1) were prepared by adding field soil 15 to 25% saturated with water to thoroughly washed "cellophane" dialyzing tubes 1⅛ in. in diameter and approximately 6 in. long. The soil was then brought to saturation with distilled water so that the soil solution would be in continuous contact with the cellophane. In addition, a 1:1 mixture by volume of distilled water and soil was sometimes used (Fig. 1). The bags were tied to a rack, openings punched in the top for gas exchange, and then thoroughly washed with a stream of sterile distilled water under pressure. After washing, the bags were dipped in a warm 1.5% agar solution containing *Fusarium* spores, and then hung in a moist chamber. After 24 to 72 hours, the bags were cut open to wash soil from inside, spread out in Petri plates, and the film was examined under the microscope. In this paper, *Fusarium* refers to *F. oxysporum* f. *cubense*.

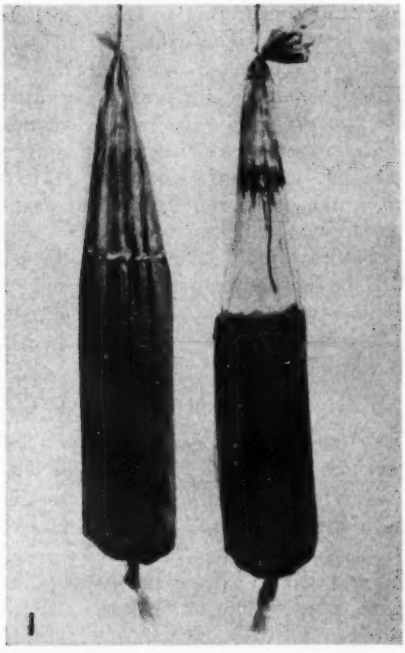


FIG. 1. Apparatus for studying effect on *Fusarium* of substances diffusing from soil through a "cellophane" membrane. The dialysis bags of cellulose film containing saturated soil (left) or a 1:1 suspension of soil and distilled water (right) were dipped in an agar spore suspension of *Fusarium* and hung in a moist chamber. At intervals up to 72 hours the bags were cut open, the soil washed out, and strips of the film laid in Petri plates for microscopic examination.

Soil-amended agar plates were prepared using a modification of the technique described by Brouwers and Bonnier (1). One gram of non-sterile or sterile soil was added to 10 ml. of warm sterile 1.5% agar in test tubes, gently shaken for 1 minute, and then poured in sterile Petri plates. During cooling, the soil settled to the bottom of the plates which were then surface streaked with water suspensions of *Fusarium* spores.

The soils used were loam and clay loam from an area described previously (18). The loam was representative of an area where bananas were destroyed by *Fusarium* wilt within 3 to 5 years after planting and the clay loam was from an area where bananas remained productive for 15 to 30 years.

As a *Fusarium* source in early experiments, 2 to 4 g. of a 2- to 6-week-old 10% corn meal-in-sand culture of *Fusarium* was mixed in 50 ml. of distilled water, the supernatant filtered through a coarse paper towel, and 0.25 to 0.5 ml. of the spore suspension added to each culture plate containing 5 ml. of extract. In later experiments, in order to avoid nutrient effects from the corn meal-in-sand, *Fusarium* spores were obtained by dragging a moistened wire loop lightly over the surface of 5- to 7-day-old colonies growing on

peptone-dextrose agar, and transferring to sterile distilled water. In most experiments freshly isolated *Fusarium* cultures from diseased bananas were used without reference to clonal characteristics. Later, it was found that there were two widely distributed clonal groups in nature, which possessed different growth and sporulating capacities (21). These clones are referred to as yellow and non-yellow in the more recent experiments reported in this paper.

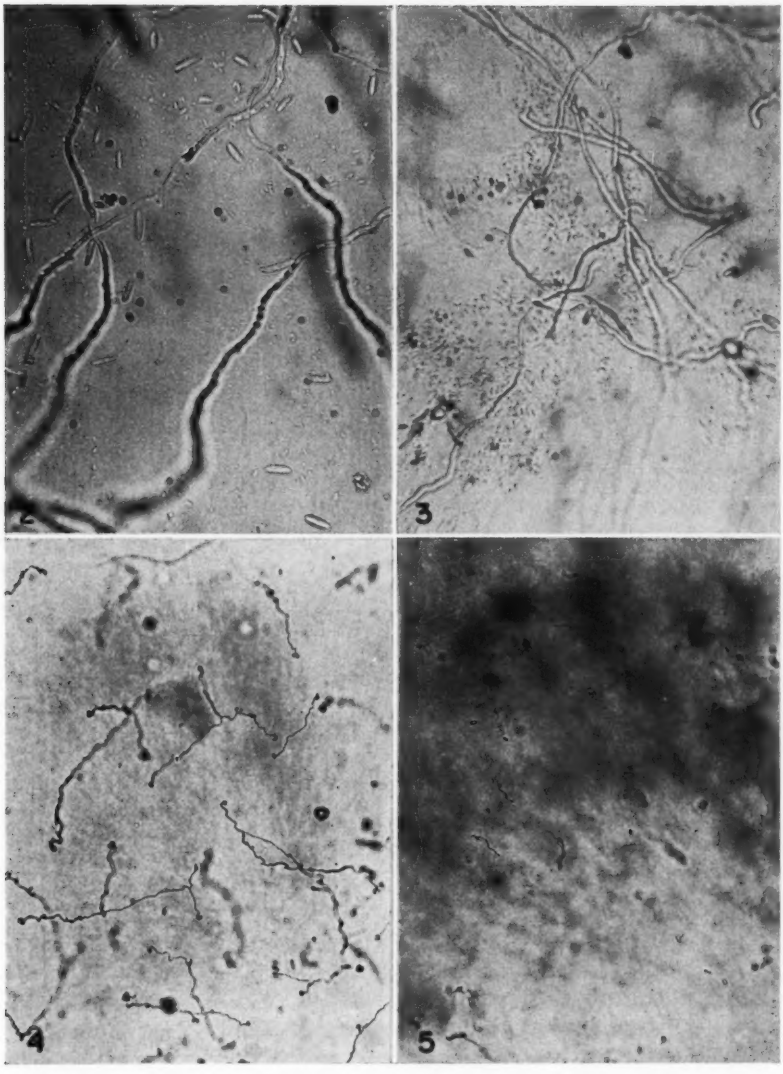
Germination, growth, and sporulation were observed in Petri plates, usually under 100× magnification, and a scoring system was used to express measurements. Usually, examination and scoring of relative growth in 15 to 25 fields of each of three replicates were sufficient to indicate differences between soil extracts studied. The sporulating potential of *Fusarium*, which is exceedingly important in epidemiology and pathogenesis, received the most attention. All experiments were undertaken at an air-conditioned room temperature of 22° to 27° C.

Results

General Growth Characteristics of Fusarium in Aqueous Soil Extracts, in Agar Films on Cellophane Soil Bags, and in Soil-Amended and Plain Agar

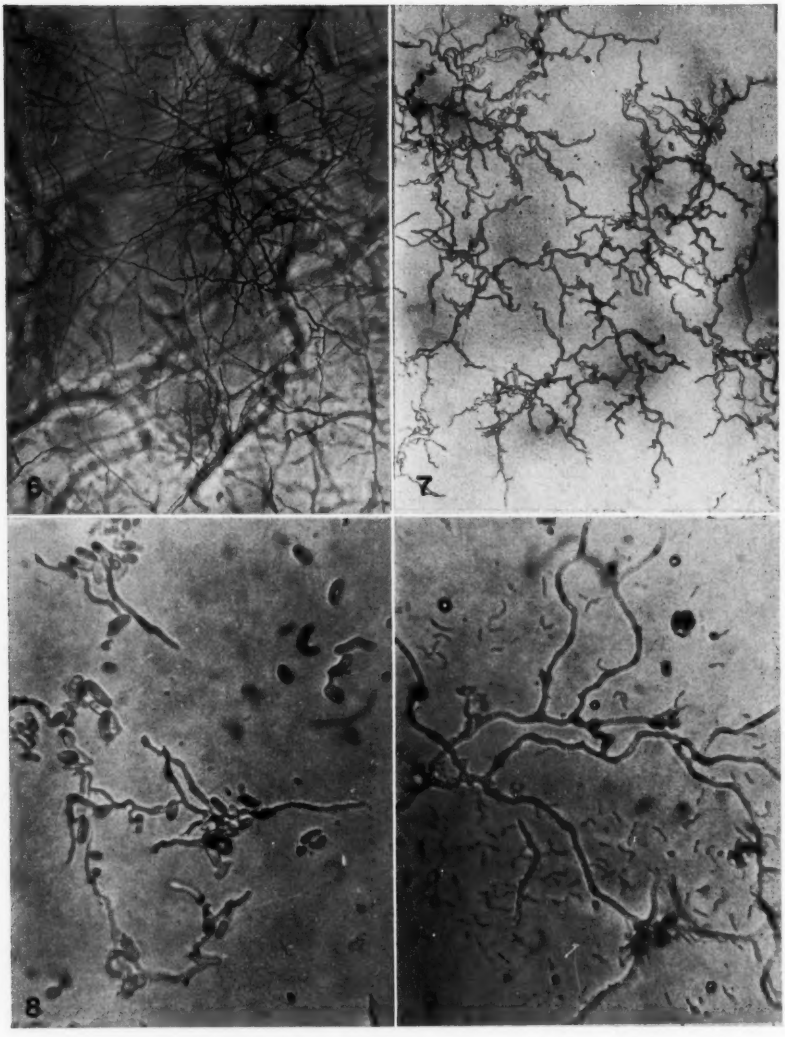
The spore suspension from corn meal-in-sand cultures added to soil extracts and to agar consisted mostly of microconidia of the non-yellow and macroconidia of the yellow race. There was little or no evidence of germ tubes before 6 hours and most began to appear between 8 and 12 hours. Where nutrition was adequate, microconidia or macroconidia were often produced within 18 hours, and where nutrition was deficient, spores were not produced until 24 to 42 hours. If spores were not produced within 42 hours they were rarely produced after 42 hours. Subsequent studies showed that rates of germination, hyphal growth, and sporulation were strongly influenced by the fungus race. In contrast to conidia, chlamydospores were rarely formed in soil extracts before 42 hours although occasionally immature chlamydospore primordia were present at 42 hours. Fully formed chlamydospores were sometimes present at 72 hours but usually were most abundant at 120 hours in aqueous soil extracts with added nutrients. Chlamydospore formation was much more variable than conidial formation, even between extracts from the same soil. It appeared that chlamydospore formation was more sensitive to the extract than was conidial formation. In general, however, hyphal chlamydospores and conidia were produced sooner and more abundantly in loam than in clay loam extracts. Usually, where conidial production was abundant (Fig. 2), chlamydospores were formed late and in small numbers or not at all. Sometimes conidia were not produced even though hyphal growth was abundant (Fig. 3), and chlamydospores formed after 4 to 5 days.

Other fungi or actinomycetes rarely grew in the extracts during the 42 to 72 hours' duration of most experiments. However, bacteria and protozoa were present and multiplied, bacterial clumps often being concentrated adjacent to the fungal hyphae (Fig. 3). Growth and sporulation of *Fusarium* could not be correlated with bacterial numbers, which were often large in extracts with added nutrient.



FIGS. 2–3. Sporulation of *F. oxysporum* f. *cubense* in aqueous soil extracts from loam (Fig. 2) and absence of sporulation in clay loam (Fig. 3) even though hyphal growth was vigorous (450×).

FIGS. 4–5. Growth of the non-yellow race of *F. oxysporum* f. *cubense* in agar films on bags containing loam (Fig. 4) and non-sterile clay loam (Fig. 5) after 42 hours (150×). Note weak germ tubes and non-germinated spores on clay loam.



FIGS. 6–7. Growth of the yellow race in agar films on sterile loam soil bags (Fig. 6) and sterile clay loam (Fig. 7) after 42 hours (150×). Note extensive branching and curling of hyphae in clay loam. Compare abundance of fungus growth on the sterilized soil bags in Figs. 6–7 with paucity of growth on the non-sterilized soil bags in Figs. 4–5.

FIGS. 8–9. Characteristics of growth of non-yellow (Fig. 8) and yellow (Fig. 9) races of *F. oxysporum* f. *cubense* after 20 hours on sterile soil-amended 1.5% agar. Hyphal growth is extensive with the yellow whereas the non-yellow produces a small number of germ tubes which frequently anastomose with neighboring spores or germ tubes. Also, there is a much greater percentage of dormant microconidia with the non-yellow than the yellow race (675×).

On agar films surrounding cellophane soil bags *Fusarium* sometimes sporulated, and formed chlamydospores more rapidly than in aqueous soil extracts. Sporulation, in some cases, occurred within 24 hours and chlamydospores formed within 72 hours. In agar films on the cellophane bags that contained acid loam, hyphal growth was usually vigorous (Fig. 4) whereas hyphae usually grew poorly or not at all from microconidia on the cellophane bags containing alkaline clay loam (Fig. 5). Chlamydospore formation from conidia and hyphae sometimes occurred with yellow and non-yellow clones in agar films on cellophane in contact with acid loam but rarely with alkaline clay loam unless the latter had been sterilized. Sterilization of soil greatly stimulated growth, sporulation, and chlamydospore formation, which occurred with varying degrees of conidial sporulation. Even after sterilization, differences in amount and characteristics of *Fusarium* growth were apparent between loam and clay loam (Figs. 6–7).

The behavior of *Fusarium* on soil-amended agar plates is described in Table I. Differences in growth and sporulation between steam or ethylene oxide sterilized soil and non-sterilized soil were pronounced. There were also differences in growth between loam and clay loam, both non-sterilized and sterilized. The most important differences, however, were between clones of *Fusarium* used (Figs. 8–9). The yellow clone germinated sooner and in greater abundance, and hyphae grew more rapidly than the non-yellow clone. In addition, there was a high percentage of anastomoses between adjacent germinating microconidia (Fig. 8) of the non-yellow race. Chlamydospores formed more slowly on soil-amended and non-amended water agar than on agar on cellulose films enclosing soil, usually not appearing until after 5 to 6 days, and only on sterile soil-amended or water agar. On non-sterile soil-amended agar a few macrospores were produced on short germ tubes by the non-yellow clone on loam soil only. Also, occasional microconidia were formed by the yellow clone on loam but not on clay loam. On non-amended 1.5% water agar macroconidia and microconidia were produced abundantly by both clones, and chlamydospores began to appear in numbers after 5 to 6 days.

Effect of Different Treatments on Behavior of Fusarium in Aqueous Soil Extracts and Perfusates

The data in Table II are representative of results from 2 years' experimentation. These data show that non-sterile extracts from loam supported more germination, growth, and sporulation than those from clay loam. The addition of dextrose to non-sterile extracts stimulated less growth and sporulation in clay loam than in loam. Steam sterilization of extracts greatly stimulated growth and sporulation and tended to eliminate or reduce differences between extracts, possibly by inactivating some heat-labile inhibitor or releasing some additional nutrients.

The effect of adsorption by charcoal of these fungitoxic substances was studied using extracts from soils of different moisture content. The soils extracted had been maintained for 3 to 4 weeks air-dry, and at moisture levels

TABLE I

RESPONSES OF YELLOW AND NON-YELLOW RACES OF *Fusarium oxysporum* f. *cubense* ON 1.5% WATER AGAR TO DIFFERENT SOIL AMENDMENTS

Responses described	Soil type amendment	Agar amended with: Sterile soil — Yellow	Sterile soil — Non-yellow	Non-sterile soil — Yellow	Non-sterile soil — Non-yellow
% Germination and hyphal growth in microns at 12 hours	Loam	95* 500*	65 125 Many anastomoses	50 250	25 15
	Clay loam	95* 375*	60 125 Anastomoses	40 125	20 10
Hyphal growth in microns at 12 hours	Loam Clay loam	5000 2500	875 750	375 250	50 20
Sporulation, 24 hours	Loam	Few microconidia	Few microconidia	0	0
	Clay loam	Few microconidia	Few microconidia	0	0
Sporulation, 42 hours	Loam	Abundant microconidia; few macroconidia	Abundant macroconidia; few microconidia	Very few microconidia	Few macroconidia
	Clay loam	"	"	0	0
Chlamydospore production, 6 days	Loam	Beginning to form	Beginning to form	0	0
	Clay loam	Beginning to form	Beginning to form	0	0

*Upper figures, percentage germination; lower figures, average growth of germ tubes in microns.

of 25 and 75% of saturation. To each 100 ml. of clear extract was added 0.5 g. of activated charcoal (Norit), then the extract was shaken briefly and filtered. This activated charcoal treatment permitted increased growth and in some cases sporulation in the extracts (Table III). Also extracts from air-dry soil supported greater growth, and in some cases sporulation, than extracts from soils 25–75% saturated. Charcoal treatment reduced, but did not eliminate, differences, particularly in sporulation, between loam and clay loam extracts used. The effect of soil moisture on extract characteristics was studied further in a series of experiments using two sources of nutrient (Table IV). The data confirmed previous observations that extracts from air-dry soil were less inhibitory to growth than extracts from soil 25% saturated with water. The greatest difference in inhibition between extracts from dry and moist soil was with clay loam where germination was often inhibited. Germination and hyphal growth in loam extracts were stimulated by concentrations of 0.01% banana root sap and 0.1% dextrose.

TABLE II

GROWTH AND SPORULATION OF *Fusarium oxysporum* f. *cubense* AFTER 48 HOURS IN STERILE AND NON-STERILE AQUEOUS EXTRACTS FROM DIFFERENT SOILS

Soil extract sterilized	Extract from soil type	Dextrose added to extract, %	Germination, %	Hyphal growth*	Sporulation†
No	Loam	0.0	90	4	+
		0.2	100	7	++
	Clay loam	0.0	10	2	0
		0.2	100	3	+
Yes	Loam	0.0	100	9	++
		0.2	100	10	+++
	Clay loam	0.0	100	9	++
		0.2	100	10	+++

*Hyphal growth scored from 0 to 10 maximum.
†Sporulation scored: 0 none; + trace; ++ abundant; +++ very abundant.

TABLE III

EFFECT OF MOISTURE LEVEL IN STORED SOIL AND OF CHARCOAL TREATMENT OF THE AQUEOUS SOIL EXTRACTS ON 48-HOUR GROWTH AND SPORULATION OF *Fusarium oxysporum* f. *cubense*

Treatment of soil extract	Extract from soil type	Moisture level of soil during storage					
		Air-dry		25% saturation		75% saturation	
		Growth*	Sporulation†	Growth	Sporulation	Growth	Sporulation
Control	Loam	8	+	3	0	1	0
	Clay loam	2	0	1	0	1	0
Activated charcoal treatment	Loam	10	++	6	0	2	0
	Clay loam	9	0	2	0	2	+

*Hyphal growth scored from 0 to 10 maximum.
†Sporulation scored: 0 none; + trace; ++ abundant; +++ very abundant.

TABLE IV

EFFECT OF MOISTURE LEVEL IN STORED SOIL ON 24-HOUR GERMINATION AND HYPHAL GROWTH OF *Fusarium oxysporum* f. *cubense* IN AQUEOUS SOIL EXTRACTS

Nutrients added to soil extract	Extract from soil type	Moisture level of soil during storage: Air-dry, Germination, %	Air-dry, Hyphal growth*	25% saturation, Germination, %	25% saturation, Hyphal growth
None	Loam	90	4	75	3
	Clay loam	20	2	1	2
Banana root sap, 0.01%	Loam	100	6	80	4
	Clay loam	40	2	0	0
Dextrose, 0.1%	Loam	100	10	90	8
	Clay loam	60	6	1	3

*Hyphal growth scored from 0 to 10 maximum.

TABLE V

EFFECT OF TREATMENT WITH HEAT AND SEITZ FILTRATION WITH AND WITHOUT DEXTROSE ON GROWTH, SPORULATION, AND CHLAMYDOSPORE FORMATION BY *Fusarium oxysporum* f. *cubense* AFTER 4 DAYS IN AQUEOUS SOIL EXTRACTS

Treatment	Hyphal growth*		Sporulation*		Chlamydospores*	
	Loam	Clay loam	Loam	Clay loam	Loam	Clay loam
No dextrose						
Control	2	0	0	0	++	0
Heat sterilized	5	3	+	0	+	0
Seitz filtered	4	2	++	0	0	0
Dextrose, 0.2%						
Control	6	3	++	0	+++	0
Heat sterilized	10	8	+++	+	+++	+
Seitz filtered	8	5	+++	0	+	0

*Hyphal growth scored from 0 to 10 maximum; sporulation and chlamydospores scored: 0 none; + trace; ++ abundant; +++ very abundant.

TABLE VI

EFFECT OF TREATMENT WITH HEAT, CHARCOAL, AND SEITZ FILTRATION WITH AND WITHOUT DEXTROSE ON GROWTH AND SPORULATION BY *Fusarium oxysporum* f. *cubense* AFTER 24 HOURS IN AQUEOUS SOIL EXTRACTS

Treatment	Germination, %		Hyphal growth*		Sporulation*	
	Loam	Clay loam	Loam	Clay loam	Loam	Clay loam
No dextrose						
Control	0	0	0	0	0	0
Heat sterilized	50	50	0.5	0.2	0	0
Charcoal filtered	10	5	0.2	0.1	0	0
Seitz filtered	100	55	1	0.2	0	0
Dextrose, 0.2%						
Control	100	100	1.8	1.5	++	0†
Heat sterilized	100	100	10	10	+++	++
Charcoal filtered	100	100	2.5	2.0	+	0†
Setiz filtered	100	100	4.5	2.0	++	0†

*Hyphal growth scored from 0 to 10 maximum; sporulation scored: 0 none; + trace; ++ abundant; +++ very abundant.
†Spores present after 42 hours.

The influence of heat sterilization, of Seitz filtration, and of adding dextrose on the behavior of *Fusarium* in extracts of both soils was studied (Table V). Heat sterilization and Seitz filtration increased growth in loam and clay loam extracts. In clay loam extracts traces or no sporulation occurred in this series of experiments. The effects of sterilization and charcoal and Seitz filtration were studied further (Table VI) and previous findings that soil extracts contained fungitoxic substances which were reduced by heat sterilization, charcoal treatment, or Seitz filtration were verified. However, expression of the fungitoxic effect is strongly influenced by nutrition. In addition to the reduction or elimination of fungitoxic substances an adequate nutritional level is often required to induce sporulation. Even with a nutritional stimulus sufficient to induce sporulation in loam extract, sporulation did not occur in clay loam extract until 42 hours' incubation and was less than in loam extract.

The pH of extracts from loam and clay loam ranged from 4.6 to 6.6 and 6.6 to 7.4, respectively. Therefore, the influence of pH on fungus behavior in soil extracts was tested (Table VII). The data indicated that pH was not a direct factor causing differences in growth and sporulation between extracts. The greater fungitoxic effect on growth and sporulation of *Fusarium* in extracts of clay loam than in extracts of loam is evident in the data of Table VII. In addition, dextrose caused a stimulatory effect.

TABLE VII

EFFECT OF pH ON GROWTH AND SPORULATION OF *Fusarium oxysporum* f. *cubense* IN AQUEOUS SOIL EXTRACTS AFTER 48-HOURS' INCUBATION

Extract and amendment	pH	Hyphal growth*		Sporulation*	
		Control	0.2% dextrose	Control	0.2% dextrose
Loam	4.6	1.7	10	0	++
Loam + $Ca(OH)_2$	6.5	2.7	8	0	++
Loam + $Ca(OH)_2$	7.2	2.5	8	0	+++
Loam + NaOH	6.2	2.7	9	0	++
Loam + NaOH	7.1	2.5	8	0	+++
Clay loam	6.8	1	3	0	0
Clay loam + H_2SO_4	4.8	1	3	0	0
Clay loam + H_2SO_4	3.5	0.5	3	0	0

*Hyphal growth scored from 0 to 10 maximum; sporulation scored: 0 none; + trace; ++ abundant; +++ very abundant.

Since a nutritional stimulus was frequently required to produce sporulation in soil extracts, an experiment was conducted to test effects on sporulation of nutrients diffusing from washed and wounded banana roots (Table VIII). Wounded banana roots stimulated sporulation in soil extracts, and this stimulation was greater in loam than clay loam. Stimulation was usually most pronounced where the wounded root was exposed than when it was sealed with paraffin wax.

The effect on *Fusarium* of a 48-hour soil perfusate from loam and clay loam is shown in Table IX. Inhibition of germination and growth in clay loam is, as with filtered soil extracts, much greater than in loam. Glutamic acid greatly stimulated growth and sporulation. Sporulation occurred only in loam perfusates, however.

TABLE VIII

EFFECT OF BANANA ROOT PIECES ON GROWTH AND SPORULATION OF *Fusarium oxysporum* f. *cubense* AFTER 42 HOURS IN AQUEOUS SOIL EXTRACTS

Saturation of soil, %	Extract from soil type	Control		Root ends waxed†		Root ends open†	
		Hyphal growth‡	Sporulation‡	Hyphal growth	Sporulation	Hyphal growth	Sporulation
0*	Loam	8	+	9	++	10	+++
	Clay loam	2	0	3	+	4	+
25	Loam	6	0	8	++	7	+++
	Clay loam	1	0	2	0	2	+

*Air-dry soil.
†Three pieces of main roots of Gros Michel bananas, 1½ in. long, placed in each Petri plate containing 3 ml. of extract; freshly cut ends on one series were dipped in paraffin wax before insertion.
‡Hyphal growth scored from 0 to 10 maximum; sporulation scored: 0 none; + trace; ++ abundant; +++ very abundant.

TABLE IX

GROWTH AND SPORULATION OF *Fusarium oxysporum* f. *cubense* AFTER 3 DAYS IN DIFFERENT 48-HOUR SOIL PERFUSATES WITH AND WITHOUT GLUTAMIC ACID

	Perfusate from:	Germination, %	Hyphal growth*	Sporulation*
Control	Loam	80	2	0
	Clay loam	0	0	0
0.1% glutamic acid	Loam	90	10	+++
	Clay loam	70	8	0

*Hyphal growth scored in units from 0 to 10 maximum; sporulation scored: 0 none; + trace; ++ abundant; +++ very abundant.

Table X presents the results of a study of the effects of sterilization by fumigation on subsequent fungus behavior in a 48-hour soil perfusate. The perfusate was placed in a 2-liter flask; 1 ml. of ethylene oxide was added to a small beaker in the flask, which was stoppered for 8 hours and then opened and aerated. Fumigation with ethylene oxide greatly stimulated subsequent *Fusarium* growth, and sporulation when a nutritional stimulus was also provided.

The importance of nutrition in growth and sporulation was investigated in a series of experiments in combination with a study of the effect of passing the extracts through a fine sintered-glass filter which removed most bacteria. Data from a representative experiment in Table XI indicate that competition, and possibly antagonism, from bacteria influence response of *Fusarium* to a nutritional stimulus. Yeast extract stimulated abundant bacterial growth in the perfusates used, and there was no *Fusarium* sporulation; however, when filtered through sintered glass, the same amount of yeast extract stimulated sporulation (Table XI). Growth stimulation by 10 p.p.m. of nutrient was tested and only glutamic acid stimulated sporulation in non-filtered perfusates, whereas yeast extract and glutamic acid were stimulative in filtered perfusates.

TABLE X

GROWTH AND SPORULATION OF *Fusarium oxysporum* f. *cubense* AFTER 48 HOURS IN SOIL PERFUSATES SUBJECTED TO SEITZ FILTRATION AND FUMIGATION WITH ETHYLENE OXIDE

Perfusate treatment	Perfusate from soil type	Control		0.1% Glutamic acid	
		Hyphal growth*	Sporulation*	Hyphal growth	Sporulation
None	Loam	12.5	0	250	++
	Clay loam	0	0	125	+
Seitz filtered	Loam	25.0	0	1,875	+++
	Clay loam	12.5	0	1,875	++
Ethylene oxide fumigated	Clay loam	375	0	12,500	+++
	Clay loam	250	0	5,000	+++

*Total hyphal growth in microns in 25 fields; sporulation scored: 0 none; + trace; ++ abundant; +++ very abundant.

Evaporation of extracts in cellophane bags in front of a fan and air evaporation to dryness in open dishes reduced fungitoxic activity. *Fusarium* growth was increased in extracts subjected to such treatments and then brought up to the original volume with distilled water. Dialysis of extracts in the apparatus of Hanke and Koessler (7) for 24 hours greatly stimulated growth. However, water around the cellophane dialyzing sack reached a temperature of 56° C., which might have influenced fungitoxic activity.

A comparison of *Fusarium* growth developing in the 48-hour soil perfusates, in ethyl alcohol displaced soil solution, and in soil extracts (obtained by mixing soil and water and filtering) did not show any consistent trends except that fungus inhibition was frequently greater in soil perfusates. Studies were begun on the effect of duration of perfusion on perfusate characteristics. It was found that perfusing for 4, 6, 21, 30, and 48 hours did not seem as important as the uncontrolled variables within soil samples, for it was these variables which exerted the greatest influence on perfusate characteristics and *Fusarium* growth.

To determine if inhibitory substances in soil extracts were specific to *F. oxysporum* f. *cubense*, spores from corn meal-in-sand cultures of other fungi were added to loam and clay loam extracts. The following fungi exhibited a differential growth response similar to *F. oxysporum* f. *cubense*: *F. oxysporum* f. *lycopersici*, *F. oxysporum* f. *nicotianae*, *F. oxysporum* f. *vasinfectum*, *Aspergillus terreus*, *A. fumigatus*, and *Gliocladium deliquenscens*. It was noted that *Fusarium solani*, *Aspergillus* sp., and *Graphium fructicolum* exhibited a weak differential response to extracts from loam and clay loam.

To determine if the inhibitory substances were diffusible, soils and suspensions of soil and water were placed in cellophane soil bags as previously described (Fig. 1). After 24 to 72 hours, the cut-open bags were examined under the microscope (Figs. 4–7). Substances diffused into the agar film on the

TABLE XI

EFFECT OF NUTRITION AND SINTERED-GLASS FILTRATION ON GROWTH AND SPORULATION OF *Fusarium oxysporum* f. *cubense* IN SOIL PERFUSATE

	Development after hours in soil perfusates									
	17*		24				42			
	Hyphal growth†		Hyphal growth		Sporulation‡		Hyphal growth		Sporulation	
Nutrients, p.p.m.	Not filtered	Filtered	Not filtered	Filtered	Not filtered	Filtered	Not filtered	Filtered	Not filtered	Filtered
Control	0–0.2	1.7	0–0.2	3.5	0	0	1.0	3.5	0	0
Yeast ext. 100	2.5	5.5					2.5	10.0	0	+++
Yeast ext. 10	1.5	3.5	1.5	9.0	0	++	1.5	9.0	0	++
Glutamic acid 100	4.5	4.5					10.0	10.0	+	+
Glutamic acid 10	4.0	4.0	6.0	7.0	++	++	6.0	7.0	++	+++
Banana sap 100	1.0	1.7					4.5	9.0	+	++
Banana sap 10	1.0	1.0	1.0	1.5	0	0	3.0	5.0	0	0
Dextrose 10			1.7	4.5	0	0				

*No sporulation present at 17-hour reading.
†Hyphal growth scored from 0 to 10 maximum.
‡Sporulation scored: 0 none; + trace; ++ abundant; +++ very abundant.

cellophane soil bags and inhibited *Fusarium* growth. Treatments such as sterilization and fumigation, which destroyed most of the fungitoxic factor in soil extracts in culture dishes, acted in a similar way on the substances diffusible from soil through the cellophane membrane. Nutrients from soil also diffused through the membrane, and as a result growth on agar films on cellophane bags containing sterile soil was about 10 times greater than growth on agar films on cellophane bags containing water. It was observed when a soil suspension was added to the bags that *Fusarium* growth was less in the agar film on that portion of the membrane in contact with soil than on the portion in contact with the solution above the soil.

Discussion

Hessayon (8) defined a fungitoxin as a substance exerting an inhibitory effect at some stage of the fungal life cycle. Apparently there was a diffusible fungitoxin in the soils and soil extracts studied which was associated with bacterial and possibly other microfloral components. All factors reducing, eliminating, or altering the bacterial flora such as heat sterilization, Seitz and sintered-glass filtration, or fumigation greatly enhanced *Fusarium* growth in extracts. Other fungi or actinomycetes were not observed to grow in the extracts. Even with removal of all or most bacteria by filtering, differences in *Fusarium* behavior in the extracts of loam and of clay loam were still apparent. This indicated the presence of extracellular diffusible fungitoxins, which was proved by use of cellophane dialysis tubes. Most fungitoxic effects were destroyed by heat sterilization. However, small differences in growth and sporulation between heat-sterilized loam and clay loam extracts suggests the presence of a heat-stable weak fungitoxin in addition to a heat-labile strong fungitoxin. Fungitoxic factors present in soil extracts were not specific for one species but affected in varying degrees several fungal genera and species. In contrast to these results, Hessayon (8) found no indications of fungitoxins in aqueous soil extracts, and little direct evidence that bacteria produce fungitoxins in soil. He was able to obtain a fungitoxin in an ether extract from soils (9). Only water extracts and diffusates were used in studies reported in this paper because the soil solution is the natural solvent and mode of solute transport in soil.

Altering extract pH did not change relative fungitoxic properties. This contrasts with effects of altered soil pH on *Fusarium* sporulation (18, 19). Alteration of the pH in a soil several months prior to extraction should encourage a change in flora not likely to occur during the short-time exposure of soil extracts to pH change.

The fungitoxic activity was strongly influenced by air-drying soil. These observations are similar to those made by Newman and Norman (13) and Greig-Smith (6). Dobbs and Hinson (5) also found that prolonged drying of soils reduced a widespread soil fungistasis, which they neatly demonstrated by use of buried cellophane envelopes. It is unlikely that the nutritional influence of drying as shown by Stevenson (16) could account for all of the fungus response observed. Apparently agar does not inactivate the fungitoxin, so that soil-amended agar plates can be used to readily demonstrate soil fungitoxins.

Jefferys and Hemming (11) found that a fungitoxin diffused from soil into agar slabs. It is likely, therefore, that soil colloids do not inactivate the widespread soil fungitoxic effect. This characteristic is unlike the reported rapid adsorption of many antibiotics by soil colloids.

Nutrition is the key factor in growth and sporulation of *Fusarium* in soil extracts or diffusates, and can even mask fungitoxic effects. Depending on fungitoxic effects exerted, as little as 10 p.p.m. of a nutrient such as glutamic acid or in some cases 4% carbon dioxide in air (19, 20) can stimulate growth and sporulation. The importance of the rhizosphere in overcoming fungitoxic effects by exerting a strong nutritional influence is suggested by recent work of Buxton (2) and Jackson (10). In the above studies, banana roots also exerted a nutritional stimulus on spores present in soil extracts thus helping to overcome inherent fungitoxic properties of soil extracts. One of the most intriguing problems encountered was to ascertain the effect of nutrition and of fungitoxin acting independently on hyphal growth, conidial production, and chlamydospore formation. It was often observed that hyphal growth was abundant and vigorous but few or no conidia or chlaymdospores were produced. In contrast, abundant conidia were sometimes produced by a few hyphae. Variation in conidial production in early phases of these investigations may have been related in part to the use of different *Fusarium* clones since cultures were frequently renewed from diseased plants. Only in the latter part of these investigations, with the discovery of the yellow and non-yellow clonal groups (21), did the importance of clones and their influence on *Fusarium* behavior become evident. Chlamydospore production, which usually took three to four times as long as conidial production, was the most variable morphogenic response and is apparently most sensitive to environmental conditions. Chinn (4) obtained data, which were supported by the writer (18, 19) in studies with *F. oxysporum* f. *cubense*, showing that most fungus spores added to soil either do not germinate or germinate poorly except when a nutritional stimulus is present. However, germination and sporulation readily occurred in sterilized soil usually without added nutrient.

Park (14) studied the behavior of several fungi in an artificial soil solution and in displaced soil solutions sterilized by Seitz filtration. Among these, *F. oxysporum* f. *cubense* grew poorly in the displaced soil solution, even when enriched with 0.05 g./l. of dextrose, while good growth was obtained in an artificial soil solution. However, heat sterilizing the displaced soil solution resulted in as good a growth as occurred in the artificial soil solution. Park attributed this behavior to the presence in the displaced soil solution of a naturally occurring toxin easily destroyed by heat. In a subsequent paper Park (15) described an important experiment in which displaced soil solutions were left exposed to air. Those diluted 1:3 and 1:4 contained small fungal colonies after 21 days. Another series was autoclaved and Seitz filtered and colonies developed on these after 6 days. Park interpreted these results as indicating that the soil solution contains sufficient nutrients to support growth of fungi when inhibitory substances are removed by heat, Seitz filtering, or diluting with water. The conclusions of Park are supported by our data.

Likais (12) described inhibitory substances in soil extracts tested against *Pythium de Baryanum* in Knopf nutrient solution, and stated that disease incidence could be decreased by leaching a fungitoxin from the soil or increased by adding the leachate containing the toxic principal to soil. No figures are given to substantiate these results, and distinctions are inadequate between effects of leaching on nutrition, the physical, microbiological, and environmental status of the soils and effects of removal of a toxic substance in the leachate.

Research on soil fungi in general indicates that fungus growth and sporulation in soil is dependent on two interacting factors: soil fungitoxins of biological origin present in the soil solution, and nutrition. Adequate nutrition may overcome fungitoxin effects, and inadequate nutrition may be compensated for by a lesser fungitoxin effect.

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THE EFFECT OF DARKNESS ON LESION PRODUCTION IN NICOTIANA GLUTINOSA L.[1]

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Abstract

Comparison, in the dark and light, of *N. glutinosa* plants inoculated with TMV indicates increased susceptibility in the dark. Rubbing with carborundum produces injury lesions similar to virus lesions, and so may give misleading results in virus assay studies.

Incidental to a study of the respiration of *Nicotiana glutinosa* leaves inoculated with tobacco mosaic virus (TMV), we observed some interesting differences in the local lesion response when some of these leaves were kept in the dark and others in the light.

Certain sets of *N. glutinosa* plants were kept in a dark chamber at a constant temperature of 76° F. for 48 hours before, and 24–36 hours after, inoculation. Similar sets of plants were kept at the same temperature in a lighted chamber (1200 ft-c. for 15 hours a day) for 48 hours before, and 24–36 hours after, inoculation. These plants will be referred to as dark plants and light plants respectively. After the initial treatment of 48 hours of dark or light, all the plants were lightly dusted with carborundum. A set of dark plants and a set of light plants were then inoculated with purified TMV, and another set each of the dark and light plants were rubbed with water.

Twenty-four to thirty-six hours after inoculation the TMV-inoculated dark plants showed numerous local lesions far in excess of those produced on the light plants. Furthermore, the lesions on the dark plants were grayish in color, and tended to coalesce, while the lesions on the light plants were typically brown and discrete.

After the same interval, the dark plants that had been rubbed with water also produced local lesions that were indistinguishable from the lesions on the dark plants inoculated with TMV. The similarity of the lesions produced by water and by the virus was amply demonstrated by several unsuspecting but otherwise competent observers who mistook both sets for TMV lesions.

It was possible to demonstrate in several ways that the large number of lesions produced in darkness was primarily a result of rubbing injury that developed in the dark. When the dark plants were rubbed with water, but *without* carborundum, no lesions were produced at all. Furthermore, the number of lesions produced by rubbing the dark plants in the presence of carborundum was also shown to be dependent on the extent of the dark treatment. When a period of darkness both preceded and followed rubbing with water, more lesions were produced than when rubbing was preceded by light treatment and followed by a period of darkness. The least number of lesions in the presence of carborundum was produced when the leaves were exposed to darkness first, then rubbed with water, and then exposed to a period of light.

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It appears, then, that, in addition to the essential requirement of injury with an abrasive, the production of such virus-like lesions depends more on the period of darkness after rubbing than on the period of darkness before rubbing.

However, although the great excess of lesions produced on the dark plants was mainly the result of rubbing injury, dark treatment could be shown, under certain conditions, to increase susceptibility to virus infection. Since the appearance of the lesions on the dark plants inoculated with TMV was indistinguishable from the appearance of the lesions on the dark plants rubbed with water, it was impossible to say which were virus lesions and which were injury lesions on the TMV-inoculated dark leaves. But when the leaves were allowed to dry thoroughly, sharp differences emerged, for the lesions on the water-rubbed dark leaves disappeared on the dried leaves, whereas lesions (somewhat indistinct) could still be seen on the TMV-inoculated, dried dark leaves. This treatment, then, made it possible to distinguish the virus lesions from the injury lesions.

It was found, after drying of the leaves, that when inoculation was made with the aid of carborundum the number of lesions produced by the virus on the dark plants was actually far smaller than the number produced on the light plants. However, on each occasion when the plants were inoculated with TMV but without carborundum, the dark plants produced twice as many lesions as did the light plants (e.g., in one experiment, 298 and 116 lesions respectively). It is probable that this reversal in the number of lesions produced with and without carborundum, under our conditions, is related to the injury caused by the abrasive in the dark. Thus, if leaves are inoculated with TMV *and* carborundum, in the dark, so much injury is induced by abrasion that the inoculated cells die without permitting the virus to reproduce and form a true virus lesion. This would account for the observation that the number of *virus* lesions on the dark leaves is far smaller than that on the light leaves. On the other hand, it was observed that, when abrasion is omitted, more virus lesions are produced on the dark than on the light plants, and so one can conclude that, in the absence of the confusing injury factor, dark treatment does actually increase the susceptibility of *N. glutinosa* leaves to TMV infection.

The relevance of these observations to studies of environmental factors on lesion production, such as that of Matthews (1), needs hardly to be pointed out. Furthermore, the rubbing injury in darkness by water also assumes significance in respiration studies, since the respiration of such injured plants is increased to about the same level as dark leaves inoculated with TMV, in contrast to results obtained with light plants, where rubbing with water does not affect respiration (2). Details of these respiration experiments will be given in a later publication.

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INHIBITION SÉLECTIVE DE LA PHOTOSYNTHÈSE PAR LE MÉTHANOL CHEZ SCENEDESMUS[1]

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Abstract

In the presence of methanol at concentrations up to 12% by volume, photosynthetic fixation of $C^{14}O_2$ into glycolic acid, glycine, alanine, serine, and an unknown compound proceeds at nearly normal rates over periods of 1 to 8 minutes. However, fixation into other compounds, such as phosphoglyceric, phosphopyruvic, and aspartic acids and the lipids, is suppressed over induction periods of several minutes. The results indicate that methanol inhibits the photosynthetic cycle, thus unmasking an alternative path of fixation into the simple acids. It is suggested that the initial step is the carboxylation of phosphoenolpyruvic acid.

Introduction

Les travaux de Calvin et de ses collaborateurs (1, 2, 3, 4, 5, 6) ont révélé que l'acide phosphoglycérique est le premier composé marqué dans l'assimilation photosynthétique de $C^{14}O_2$. Le cycle proposé par ces auteurs rend compte des faits observés et permet de suivre le cheminement du carbone depuis l'anhydride carbonique jusqu'au saccharose. Cependant, dans ce schéma, plusieurs composés, qui sont marqués tôt et en grandes quantités, ne sont pas considérés comme des intermédiaires mais comme des satellites de ces derniers. Tels sont, par exemple, les acides malique, aspartique et glycolique, la sérine, l'alanine et la glycine. On peut se demander s'il n'existe pas d'autres voies d'entrée du carbone dans ces composés. On sait déjà que la fixation du carbone radioactif dans l'acide malique (1) peut être inhibée sans diminuer la fixation dans les autres intermédiaires. D'autre part, le dinitrophénol semble inhiber sélectivement la fixation dans l'acide phosphoglycérique (12). La fixation dans la glycine, la sérine et l'alanine n'est pas affectée par la température de la même façon que celle des autres composés (13). Enfin, il semble que les acides aspartique et glutamique ne soient pas formés comme satellites du cycle de Krebs (11).

Dans l'espoir de mettre en évidence une voie alternative d'assimilation, nous avons cherché lesquels des intermédiaires marqués résistent le mieux à des conditions fortement anormales qui aboutissent à la suppression quasi complète de la photosynthèse. Nous avons utilisé à cette fin de fortes doses de méthanol, d'éthanol et d'acétone, ainsi que des inhibiteurs puissants et l'inactivation par irradiation à la lumière ultraviolette. Le présent mémoire porte sur les résultats obtenus chez *Scenedesmus* en présence des fortes concentrations de méthanol.

Méthode expérimentale

Les algues utilisées provenaient d'une souche de *Scenedesmus obliquus* obtenue de General Biological Supply House, Inc., Chicago. Nous n'avons pas contrôlé la pureté de cette souche.

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Contribution du Département de Chimie, Université Laval, Québec, Qué.

Ces algues étaient cultivées à 22° C. à la lumière de tubes fluorescents, dans des ballons en Pyrex de 2 litres contenant 1.5 litres de milieu de culture (8) dans lequel barbotait un courant d'air filtré contenant 5% d'anhydride carbonique. À une concentration de 2 g. d'algues mouillées par litre, des échantillons de la culture étaient centrifugés et resuspendus dans de l'eau redistillée à laquelle on ajoutait du milieu de culture et du fer ferreux à des concentrations 10 fois moindres que durant la culture, de façon à ne pas gêner la chromatographie des extraits. Après une réadaptation d'une heure et demie à la lumière dans des conditions semblables à celles employées pour la culture, des échantillons de 30 cm.^{3} contenant 0.2 g. d'algues mouillées étaient irradiés dans une bouteille à faces parallèles de 10×7×2.5 cm., placée dans un bain d'eau à une distance de 15 cm. d'une lampe Photoflood No. RFL2. Au bout de 10 minutes d'adaptation à ces conditions commençait l'expérience.

Chaque expérience ou groupe d'expériences en présence d'un inhibiteur était précédée et suivie de deux expériences témoins sans inhibiteur, effectuées à quelques minutes d'intervalle avec des échantillons de la même culture. Pour chaque expérience de fixation, on injectait à l'instant zéro, au moyen d'une micropipette, 50 λ d'une solution de $Na_2C^{14}O_3$. Cette dose contenait 5 μc. Après avoir laissé les algues photosynthétiser durant des périodes allant de 0.5 à 8 minutes suivant le cas, on les tuait rapidement en versant les 30 cm.^{3} de suspension dans 110 cm.^{3} d'éthanol bouillant.

Des échantillons de 500 λ de cet extrait alcoolique, avant et après centrifugation, étaient placés et séchés sur des plats en aluminium de 3 cm. de diamètre et la radioactivité fixée, totale ou soluble, était mesurée au moyen d'un compteur de type Nuclear Corporation D-34.

Les 140 cm.^{3} d'extrait étaient concentrés jusqu'à un volume de 2.5 cm.^{3} par évaporation à 25–28° C. sous le vide d'un aspirateur. Des extraits de 50 ou 100 λ de ce concentré étaient chromatographiés sur des feuilles de papier Whatman No. 1 de 46×57 cm., en utilisant comme solvants les solutions phénol–eau et butanol – acide propionique – eau, dans les conditions décrites par Benson et ses collaborateurs (3). Un film à rayons X Kodak No-Screen était exposé durant quelques semaines à chaque chromatogramme et la radioactivité des taches repérées sur le film était mesurée au moyen d'un compteur plat Anton 1001T de 4.5 cm. de diamètre et 1 cm. de hauteur. Les taches étaient identifiées par leurs positions relatives et par des tests à la ninhydrine pour les acides aminés, au résorcinol pour le saccharose (14), ou suivant la méthode de Crowther (7) pour les phosphates organiques et celle de Morris (10) pour les protéines.

Résultats

Essais divers

Quelques expériences préliminaires montrèrent qu'il fallait de 12% à 15% en volume de méthanol pour supprimer l'assimilation photosynthétique de $C^{14}O_2$ et qu'en présence d'environ 10% de méthanol, il subsistait une fixation appréciable de la radioactivité dans les acides aminés simples.

Nous avons vérifié que l'addition de 10% de méthanol n'augmente pas sensiblement la très lente fixation de radioactivité par les algues à l'obscurité. Nous avons aussi vérifié que le méthanol n'exerce aucun effet s'il est ajouté après la période de photosynthèse, en l'incorporant à l'éthanol qui sert à tuer les algues et à en extraire le contenu. L'assimilation résiduelle observée en présence de 10% de méthanol est donc d'origine photosynthétique. Les effets constatés ne peuvent être expliqués ni par une exaltation du métabolisme obscur ni par un artéfact portant sur l'extraction.

L'inhibition causée par le méthanol est réversible dans une large mesure. Nous avons obtenu un taux de fixation de 85% de la valeur normale avec des algues centrifugées et resuspendues dans l'eau après un séjour de 2 minutes dans du méthanol à 10% où leur taux de fixation n'était que de 30% du taux normal. Les effets du méthanol ne sont donc pas attribuables à la destruction permanente de structures intracellulaires, ni à des phénomènes de coalescence (9) comme on en observe avec de grandes concentrations de certaines substances organiques.

Le tableau I présente un exemple d'analyse détaillée de la radioactivité de chromatogrammes provenant de six expériences d'une même série. Dans les trois expériences témoins, les algues ont été soumises à des périodes de photosynthèse de 4 minutes en l'absence de méthanol. La comparaison de ces témoins permet d'établir le degré de reproductibilité des résultats à l'intérieur d'un même groupe d'expériences. On voit que les fluctuations sont faibles pour des compsés tels que les acides malique et aspartique, l'alanine, la sérine et la glycine. Pour certains autres composés, elles sont largement supérieures à l'erreur statistique.

Les trois expériences en présence de 9% de méthanol ont été effectuées dans les mêmes conditions que les témoins, sauf que le méthanol a été ajouté à la suspension d'algues à des époques respectives de 2, 4 et 8 minutes avant

TABLEAU I

INFLUENCE DE CH_3OH 9% AJOUTÉ 2, 4, 8 MINUTES AVANT UNE PÉRIODE D'ASSIMILATION PHOTOSYNTHÉTIQUE DE $C^{14}O_2$ PAR *Scenedesmus* DURANT 4 MINUTES À 24° C.

Taches	Témoin	CH_3OH 9% 2 min. avant $C^{14}O_2$	CH_3OH 9% 4 min. avant $C^{14}O_2$	CH_3OH 9% 8 min. avant $C^{14}O_2$	Témoin	Témoin
Acide *p*-glycérique	365	0	0	0	343	696
Acide *p*-pyruvique	862	0	446	390	690	665
Acide aspartique	383	345	260	199	419	424
Acide glutamique	18	443	102	103	40	14
Alanine	2,628	1,007	596	509	2,727	2,746
Sérine	1,512	2,883	2,623	786	1,857	1,879
Glycine	1,090	4,163	556	185	1,276	1,191
Acide malique	2,039	1,020	664	200	1,973	1,910
Acide glycolique	916	1,511	288	231	1,136	1,715
Acide fumarique	477	276	175	88	413	312
Acide glycérique	0	0	0	0	54	111
Saccharose	2,062	2,224	569	202	3,541	3,325
Inconnu (1)	2,581	4,279	2,397	764	2,786	2,093
Total	14,933	18,151	8,676	3,657	17,255	17,081
Tache originale	11,773	10,557	5,854	2,225	11,206	12,329

NOTE: Radioactivités, en impulsions /10 minutes, des taches de chromatogrammes contenant chacun l'extrait de 4 mg. d'algues.

l'injection de $C^{14}O_2$. On voit que le méthanol exerce une forte inhibition sur la plupart des composés, mais que la glycine, la sérine et le composé inconnu subissent, au contraire, une exaltation transitoire.

Le composé désigné sous le nom d'"inconnu" n'a pu être identifié. Il se trouve sur les chromatogrammes, près de la tache originale, à une distance correspondant à $R_f \sim 0.1$ dans la direction eau–phénol. D'après les tests chimiques, ce n'est ni un acide aminé, ni un phosphate, ni un sucre, ni une protéine; nous pensons qu'il s'agit d'un polysaccharide.

Influence de la concentration du méthanol

La figure 1 montre l'influence du méthanol à diverses concentrations sur la radioactivité fixée dans les principaux composés. Dans toutes ces expériences, le méthanol était ajouté à la suspension d'algues 2 minutes avant l'injection de $Na_2C^{14}O_3$, laquelle était suivie d'une période de photosynthèse de 4 minutes. On voit que la glycine, la sérine et l'inconnu se classent dans un groupe à part par leur remarquable résistance à l'inhibition. Ils subissent même une forte exaltation aux faibles concentrations de méthanol. Ce comportement contraste avec celui des autres composés dont la radioactivité décroît rapidement avec l'augmentation de la concentration du méthanol.

Cinétique de l'assimilation

Pour comparer la cinétique d'une réaction inhibée à celle d'une réaction normale, nous avons effectué des expériences dont les résultats sont résumés

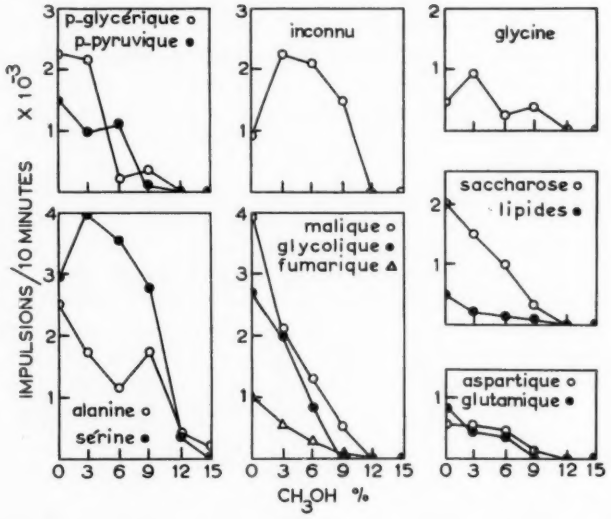


FIG. 1. Radioactivité fixée dans divers composés durant 4 minutes de photosynthèse en fonction du pourcentage en volume de méthanol. Radioactivités exprimées en impulsions $\times 10^{-3}$ par 10 minutes. Chaque chromatogramme contenait l'extrait de 4 mg. d'algues mouillées.

sur la figure 2. Pour les réactions inhibées, les algues étaient mises en présence de méthanol à 10% en volume 2 minutes avant l'injection de $Na_2C^{14}O_3$ et on les laissait ensuite photosynthétiser en présence de ce méthanol durant des périodes allant de 0.5 à 8 minutes. Toutes les expériences de cette série ont été faites sur une même récolte d'algues dans un intervalle d'environ deux heures.

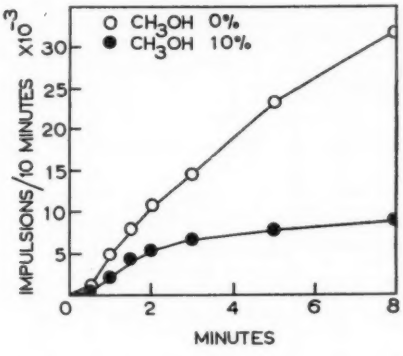


FIG. 2. Radioactivités totales (en impulsions $\times 10^{-3}$ par 10 minutes) fixées en fonction du temps en l'absence de méthanol (○), en présence de 10% en volume de méthanol (●).

L'analyse chromatographique des échantillons dont les radioactivités globales sont données sur la figure 2 a fourni les données représentées sur la figure 3. On y voit comment varie, en fonction du temps, la radioactivité fixée dans plusieurs composés dans la réaction normale et dans la réaction inhibée. On constate une parenté très nette entre les comportements de plusieurs composés. L'acide phosphoglycérique et les lipides sont supprimés au moins jusqu'à la huitième minute. Les acides phosphopyruvique et aspartique sont marqués à une vitesse normale, mais après une période d'induction de 3 minutes. L'alanine et l'acide malique subissent des inhibitions simples et sensiblement égales, sans retard. La sérine subit tout au plus un léger retard initial pour se stabiliser ensuite au même niveau qu'en l'absence de méthanol. On constate un phénomène de transition très marqué dans les cas de l'acide glycolique, dont la radioactivité baisse après la première minute, et de la glycine, qui est le siège d'une exaltation passagère précédée d'une courte période d'induction. Cependant, il ne faut pas attacher trop de signification aux comportements de l'acide glycolique et du saccharose, qui sont souvent capricieux (tableau I).

Pour essayer de mettre en évidence l'ordre dans lequel la radioactivité apparaît dans certains composés, nous avons représenté sur la figure 4 les radioactivités de certains d'entre eux en pourcentages de la radioactivité totale. En l'absence de méthanol, l'acide phosphoglycérique semble être le premier à apparaître, et cela est en accord avec les résultats de Calvin. Cependant, l'inconnu et l'acide malique le suivent de près. Par contre, en présence de

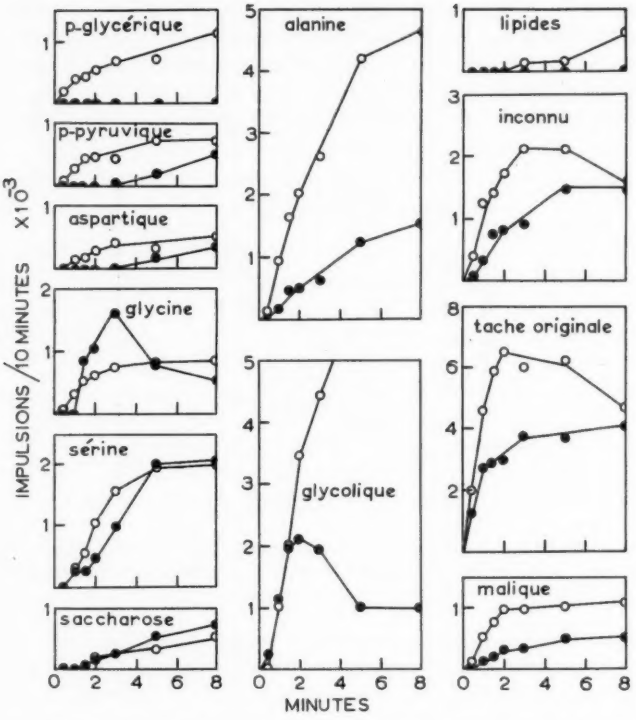


FIG. 3. Comparaison des cinétiques d'assimilation dans divers composés en l'absence de méthanol (○) et en présence de 10% en volume de méthanol (●).

méthanol à 10%, l'acide phosphoglycérique n'apparaît pas tandis que l'acide malique et l'inconnu conservent leur priorité. Cependant, pour pouvoir conclure que l'un de ces deux composés est le premier intermédiaire marqué dans ces conditions, il faudrait plus de données sur ce qui se passe dès les premières secondes. Le rebroussement des courbes de l'acide glycolique et de la sérine montre bien le danger d'illusion inhérent à cette méthode d'extrapolation à rebours. Il reste qu'à l'échelle de temps de nos expériences, nous n'avons pas décelé de composé marqué antérieur à l'inconnu et à l'acide malique, en présence de 10% de méthanol.

Quelques dégradations de la glycine obtenue dans les expériences représentées par les figures 3 et 4 indiquent (tableau II) une accumulation temporaire de la radioactivité dans le groupement carboxylique durant la période de transition qui correspond à une exaltation de l'assimilation de ce composé. Cette accumulation se produit en présence de méthanol à 10% mais pas dans les réactions témoins (0%). Il semble donc que l'excès de glycine qui apparaît vers la troisième minute n'a pas la même origine que le reste de la glycine.

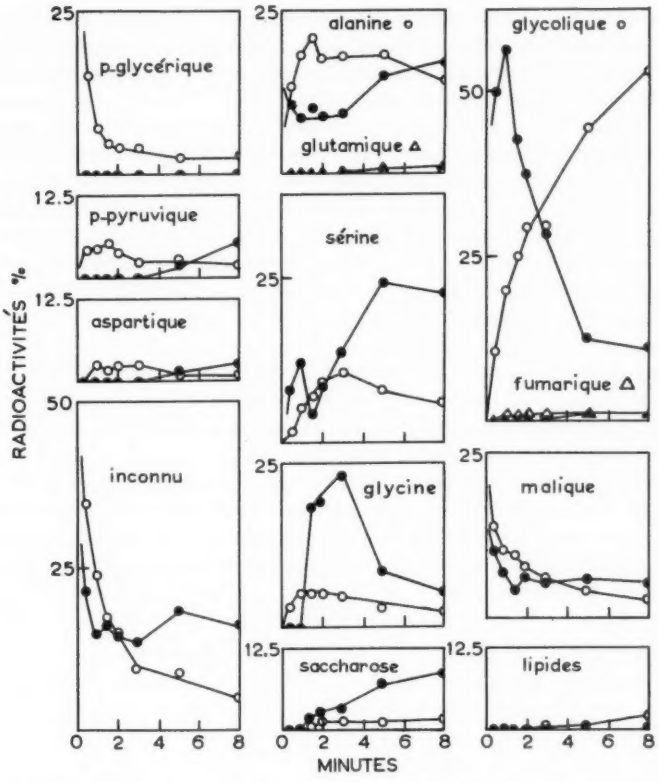


FIG. 4. Pourcentages de la radioactivité totale assimilés dans divers composés en fonction du temps en présence et en l'absence de méthanol.

TABLEAU II

DISTRIBUTION DE LA RADIOACTIVITÉ DANS LA GLYCINE À DIVERSES ÉPOQUES DE L'ASSIMILATION, EN L'ABSENCE (0%) ET EN PRÉSENCE (10%) DE MÉTHANOL

Minutes de PS	3	4	2	3	4	5	8
% de CH_3OH	0	0	10	10	10	10	10
% de la radioactivité dans —COOH	49	54	48	62	69	60	48

Discussion

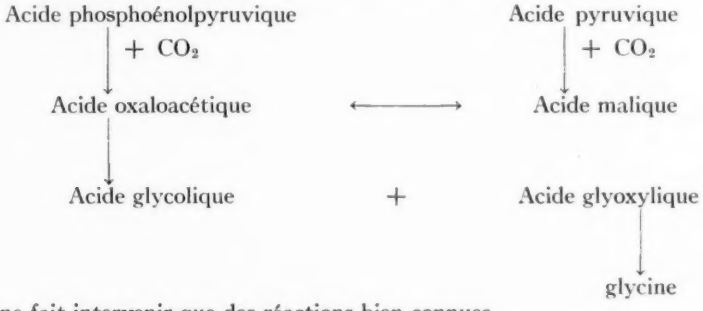
Malgré les complications qui rendent impossible une analyse détaillée de nos résultats, une conclusion générale se dégage de l'ensemble de ces expériences variées. Cette conclusion, c'est qu'on peut maintenir un taux normal de fixation photosynthétique dans des composés comme l'acide glycolique, la glycine, l'alanine, la sérine et notre composé inconnu, tout en supprimant la

fixation dans d'autres composés tels que les acides phosphoglycérique, phosphopyruvique et aspartique, ainsi que dans les lipides. Il faut donc essayer d'expliquer d'une part l'action inhibitrice du méthanol et, d'autre part, le mode de fixation dans les composés non inhibés.

L'action inhibitrice du méthanol se situe évidemment à un stade postérieur à celui de la formation de pouvoir réducteur, puisqu'il subsiste une assimilation photosynthétique considérable. Il est vraisemblable que le méthanol agisse à la manière d'un narcotique, en bloquant une réaction enzymatique, peut-être une phosphorylation. Il est possible, aussi, que le méthanol se substitue au gaz carbonique ou soit assimilé comme aliment. L'emploi de méthanol radioactif permettrait de vérifier cette hypothèse. Tous ces modes d'action conduiraient au même résultat qui est d'empêcher la fixation de la radioactivité dans certains composés.

De toute façon, le méthanol empêche le carbone radioactif d'être fixé dans l'acide phosphoglycérique et tout se passe comme si le cycle de Calvin était bloqué. Il faut donc chercher une autre voie pour expliquer sa fixation dans ceux des composés qui ne sont pas inhibés.

D'après nos courbes cinétiques (figure 4), l'apparition de l'acide malique radioactif n'est pas retardée par le méthanol. On sait déjà que ce composé ne fait pas partie du cycle de Calvin (1). Le plus simple est de supposer que cet acide malique est engendré par la carboxylation d'un composé en C_3. S'il en est ainsi, cet accepteur en C_3 ne deviendra radioactif que plus tard, par des voies indirectes. Nous suggérons que cet accepteur en C_3 est l'acide phosphopyruvique et nous proposons le schéma suivant:



qui ne fait intervenir que des réactions bien connues.

La carboxylation de l'acide phosphoénolpyruvique a déjà été invoquée pour expliquer le comportement spécial de l'acide malique (1).

Ce schéma rend compte de l'apparition hâtive de l'acide malique, peut-être directement par la réaction d'Ochoa (16), ainsi que de l'ordre chronologique et de la ressemblance des courbes d'apparition de l'acide glycolique et de la glycine. Après quelques minutes, lorsque le réservoir de l'acide malique est rempli, on devrait voir la radioactivité se répandre dans les acides phosphopyruvique, fumarique, succinique, aspartique et glutamique, ainsi que dans l'alanine et la sérine, par les voies ordinaires. C'est en effet ce qui se passe.

Cependant, la sérine et l'alanine marquées durant les deux premières minutes doivent avoir une autre origine. Une quantité importante de sérine pourrait provenir de la réaction de la glycine avec de l'acide formique (15) formé par oxydation du méthanol. Ceci expliquerait l'accumulation de sérine qui suit l'accumulation transitoire de glycine, mais n'explique pas l'apparition de la sérine durant la première minute. Cette sérine précoce pourrait être formée par carboxylation d'une petite quantité d'éthanolamine, mais nous n'avons pas de preuve de l'intervention de cette réaction. Par contre, nous n'avons pas trouvé de réaction connue à laquelle on puisse faire appel pour expliquer la formation d'alanine durant les deux premières minutes. Notons que les courbes de l'alanine et du composé inconnu (figures 1, 3 et 4) en régime inhibé se ressemblent beaucoup. On est donc tenté d'attribuer l'origine de ces deux composés à un mécanisme commun, probablement indépendant de ceux que nous avons discutés ci-dessus, et qui entrerait en jeu dès les premiers moments de la photosynthèse.

Il est probable que les réactions proposées jouent un rôle peu important dans la photosynthèse normale, où le pouvoir réducteur s'engage dans le cycle de Calvin de préférence aux autres voies possibles. Une fois ce cycle bloqué, ces autres voies seraient non seulement démasquées, mais leur importance absolue serait accrue du fait qu'elles bénéficient sans concurrence du gaz carbonique et du pouvoir réducteur disponibles.

Résumé

En présence de méthanol à des concentrations allant jusqu'à 12% en volume, la fixation photosynthétique de $C^{14}O_2$ dans l'acide glycolique, la glycine, l'alanine, la sérine et un composé inconnu persiste à un taux quasi normal durant plusieurs minutes. Par contre, la fixation dans les acides phosphoglycérique, phosphopyruvique et aspartique, ainsi que dans les lipides, est supprimée durant des périodes d'induction de plusieurs minutes. Les résultats suggèrent que le méthanol inhibe le cycle photosynthétique, démasquant ainsi une voie alternative de fixation du carbone dans les acides simples. On propose, comme première étape, la carboxylation de l'acide phosphoénolpyruvique.

Remerciements

Ce travail a été subventionné par le Conseil National de Recherches du Canada. L'un de nous (M. L.) a bénéficié d'une bourse de l'Office de Recherches scientifiques de la province de Québec. Nous remercions le Dr W. Corrivault de ses conseils et de son aide pour la culture des algues.

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THE METABOLISM OF INDOLE BY TOMATO-PLANT TISSUES AND EXTRACTS[1]

J. B. MUDD[2] AND SAUL ZALIK

Abstract

Tomato-plant tissues and homogenates were found to metabolize indole. Tryptophan and indoleacetic acid were identified as reaction products, and in some instances serine was required for indole metabolism. The pH optimum for the reaction was about 5.0. Preparations from zinc-deficient tomato plants metabolized indole more efficiently than those from normal plants.

Introduction

Skoog (9) found that, under conditions of zinc deficiency, tomato plants contained less than normal quantities of auxin. This finding was confirmed by Tsui (13), who also found that the content of tryptophan was reduced. Since tryptophan is converted to indoleacetic acid (IAA) in higher plants (15), and since the conversion of tryptophan to IAA is not affected by zinc deficiency (13), it has been concluded that zinc deficiency affects some reaction or reactions leading to the synthesis of tryptophan. There is, in fact, evidence that zinc deficiency may limit tryptophan synthesis by limiting serine formation (5).

Although the conversion of tryptophan to indoleacetic acid has been demonstrated in higher plants (3, 15), little attention has been paid to the biosynthesis of tryptophan. This is in spite of the considerable amount of work done on this problem with microorganisms. Recent reviews of these investigations have been presented by Davis (1) and Ehrensvärd (2).

Tatum and Bonner (12) found that, in *Neurospora*, tryptophan is formed by a condensation of indole and serine. Cell-free preparations of the enzyme involved in this condensation were later obtained from the same material and found to require pyridoxal phosphate as a coenzyme (14). Wildman, Ferri, and Bonner (15) have suggested that a similar mechanism may be responsible for the synthesis of tryptophan in higher plants. The work described here was designed to test this possibility. In addition, since *Neurospora* cultivated on zinc-deficient media have shown a reduced ability to effect the condensation of indole and serine (7), the effect of zinc deficiency in tomato upon this reaction was investigated.

Materials and Methods

Seeds of tomato, *Lycopersicon esculentum* (var. Bounty), were germinated in vermiculite. When the seedlings were 3–4 cm. high, they were transplanted and grown in culture solution as described by Stout and Arnon (10). Material used in the enzyme studies included cotyledons; stem slices, which were cut

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freehand; leaf disks, which were cut with a 9 mm. cork borer; and extracts of stems and leaves. The tissue extracts were prepared by grinding the plant material in distilled water and acid-washed sand in a mortar. The extract was pressed through cheesecloth and then centrifuged at 3000 r.p.m. for 30 minutes.

Enzyme assays were performed in 50 ml. Erlenmeyer flasks containing phosphate–citrate buffer, indole, serine, enzyme preparation, and distilled water to give a final volume of 20 ml. The flasks were stoppered with cotton plugs and shaken at room temperature. At intervals, 2 ml. of the reaction mixture were taken and shaken with 5 ml. of toluene to extract the residual indole. An aliquot of the toluene layer was taken for indole determination. Aliquots of the aqueous layer were assayed for either tryptophan or IAA.

Indole was determined by the method of Wood, Gunsalus, and Umbreit (16); tryptophan, by the method of Horn and Jones (4); and IAA, according to the method of Tang and Bonner (11).

Experimental Results

Metabolism of indole was catalyzed by tomato-plant cotyledons, stem slices, leaf disks, and by cell-free extracts of leaves and stems. With extracts the reaction was virtually complete after 2 hours' reaction time. With tissues the reaction was somewhat slower, presumably because of the limitation of diffusion of substrate into the cells.

Optimum pH

The pH optimum of the enzyme was determined with phosphate–citrate buffers ranging from pH 4.0 to 7.5. The reaction mixture contained 10 ml. of buffer of the indicated pH, 4 ml. of leaf extract, 3 ml. 10^{-3} *M* indole, 2 ml. 10^{-2} *M dl*-serine, and 1 ml. distilled water. Samples of the reaction mixture were taken for analyses at the start of the experiment and after 2 hours' incubation. It can be seen from Fig. 1 that the optimum pH for the metabolism or uptake of indole is about pH 5.0.

Concentration of Substrate

The effect of substrate concentration was determined in a reaction mixture containing 10 ml. buffer, 2 ml. leaf extract, varying amounts of 2.5×10^{-3} *M* indole, and distilled water to give a final volume of 20 ml. Samples of the reaction mixture were taken at the start of the experiment and after 2 hours for determination of indole concentration. From these experiments it was difficult to calculate the Michaelis constant by plotting substrate concentration directly against velocity since the maximum velocity had not been reached. Therefore, the method of Lineweaver and Burk (6) was employed. Their method involves rearranging the Michaelis–Menten equation $v = V(S)/(K_m + (S))$ to give $1/v = (K_m/V(S)) + 1/V$. Thus when $1/v$ is plotted against $1/(S)$, a straight line is obtained (Fig. 2). From the values obtained for the ordinate intercept $1/V$, and the slope K_m/V, the Michaelis constant was calculated to be 4.2×10^{-4} *M*.

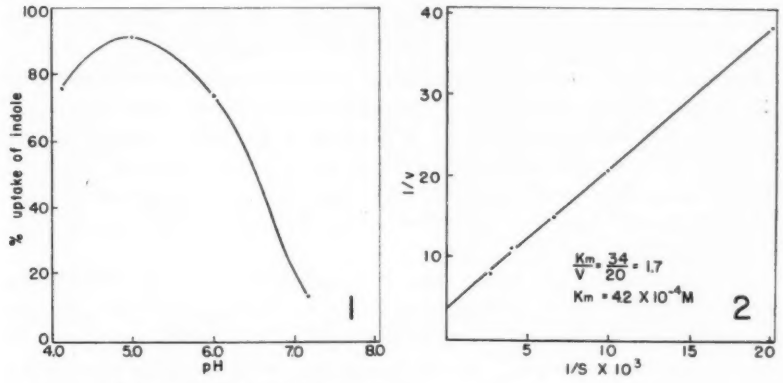

FIG. 1. Indole uptake in relation to pH.
FIG. 2. The effect of substrate concentration on the uptake of indole (Lineweaver–Burk plot). Substrate concentration S is gamma indole per ml. reaction mixture, and v is the uptake of indole in gamma per ml. per 2 hours.

Concentration of Enzyme

The effect of enzyme concentration is shown in Fig. 3. The reaction mixture contained 10 ml. buffer, 3 ml. 2.5×10^{-3} M indole, varying amounts of leaf extract, and distilled water to a final volume of 20 ml. The indole concentration was measured after a period of 2 hours. Although the reaction velocity would be expected to be directly proportional to enzyme concentration, some departure from linearity is evident. This might be attributed to insufficient substrate, particularly at higher concentrations of enzyme.

Effect of Serine Concentration

In *Neurospora*, indole is the precursor of tryptophan. Serine is necessary for this conversion. If the enzyme catalyzing the metabolism of indole in

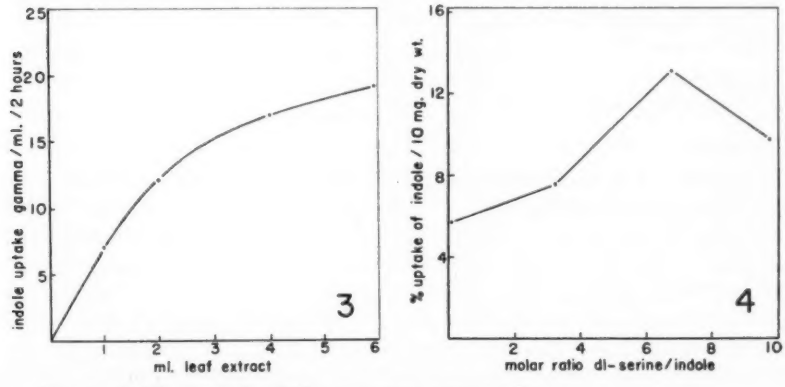

FIG. 3. Indole uptake in relation to enzyme concentration.
FIG. 4. The relation between indole uptake and serine concentration.

tomato-plant tissues is similar to the enzyme described for *Neurospora*, then serine would be expected to be required for that reaction also.

The dependency of indole metabolism on serine was demonstrated with tomato cotyledons. The reaction mixture contained 10 ml. buffer, 3 ml. 10^{-3} *M* indole, varying amounts of 10^{-2} *M dl*-serine, and distilled water to a final volume of 20 ml. Cotyledons were added as the active material, their dry weights being determined at the end of the experiment. Aliquots of the reaction mixture were taken for determination of indole concentration at the start of the experiment and after 8 hours.

It can be seen from Fig. 4 that there is an optimum serine concentration for indole metabolism when cotyledons are used as the source of the enzymes. When a similar experiment was performed with cell-free extracts, no stimulation by serine was observed. At higher concentrations there was some inhibition, thus agreeing with the trend observed using cotyledons.

Products of the Reaction

If the metabolism of indole by tomato-plant tissues and extracts is achieved by a reaction similar to the one described for *Neurospora*, it should be possible to detect tryptophan as a product. This was found to be the case, but the sensitivity of the colorimetric test for tryptophan was not great enough to estimate the formation of tryptophan quantitatively. In some experiments the formation of IAA from indole was measured. IAA was frequently detected in the reaction mixture when indole was metabolized, but the percentage of the theoretical maximum, on the basis of indole disappearance, was variable. The lowest value obtained was 20% of the theoretical maximum, but other experiments gave yields approaching 100% of the theoretical maximum.

Effect of Zinc Deficiency

When tissues and extracts of zinc-deficient plants were compared with normal plants for their efficiency in metabolizing indole, it was found, surprisingly, that the preparations from the deficient plants were more efficient. The results of several experiments are shown in Table I.

Inhibitors

The effects of three inhibitors, sodium cyanide, sodium azide, and 8-hydroxyquinoline, on the metabolism of indole were tested. The reaction vessels contained 10 ml. buffer, 4 ml. leaf extract, 4 ml. 10^{-3} *M* indole, and 2 ml. inhibitor. The extract was incubated with the inhibitor and buffer for 15 minutes before addition of the indole. Samples were taken for analyses at the start of the reaction and after 1 hour, and the concentration of indole determined. It was found that sodium cyanide at a final concentration of 10^{-3} *M* inhibited the metabolism of indole by 87%. A concentration of 5×10^{-4} *M* 8-hydroxyquinoline inhibited the metabolism of indole by 67%, and sodium

TABLE I

COMPARISON OF ZINC-DEFICIENT AND HEALTHY TOMATO-PLANT TISSUES AND EXTRACTS FOR THEIR ABILITY TO METABOLIZE INDOLE

Material		Incubation time (hours)	Gamma indole/ ml./50 mg. dry wt.	Ratio D/C*
A. Tissues				
Leaf disks	C	24	9.8	
	D		20.1	2.1
Stem slices	C	19	3.3	
	D		4.1	1.2
Stem slices	C	24	3.8	
	D		4.9	1.3
			Gamma indole/ ml./mg. protein N	
B. Extracts				
Leaf	C	2	12.4	
	D		30.5	2.5
Stem	C	2	68	
	D		93	1.4

*C—from plants grown in a complete nutrient solution; D—from plants grown in a zinc-deficient nutrient solution.

azide at 10^{-3} *M* inhibited indole metabolism by 55%. These three inhibitors are all assumed to bring about enzyme inhibition through metal inactivation. Therefore, these findings seem to imply the participation of some metallic ion.

Discussion

The observation that indole is actively metabolized by tomato-plant tissues and tissue extracts is by itself no indication that indole is a normal metabolite of the tissues of higher plants. However, the detection, in these studies, of tryptophan and IAA accumulation concurrent with the disappearance of indole is of genuine significance in relation to the synthesis of IAA in plant tissues. Also, the conversion of indole to tryptophan has important implications in the study of zinc deficiency in higher plants.

Zinc-deficient tomato plants contain less tryptophan and IAA than normal plants (9, 13), and *Neurospora* grown in zinc-deficient media have a reduced ability to synthesize tryptophan from indole (7). The results obtained with tomato tissue suggested the possibility of a pathway for indole metabolism similar to that which occurs in *Neurospora*. In *Neurospora*, zinc appears to influence the condensation of serine and indole. As in *Neurospora*, serine stimulated indole metabolism by tomato cotyledons, but contrary to the behavior of zinc-deficient *Neurospora* preparations, the tissues from zinc-deficient plants were more efficient in metabolizing indole than were those from normal plants. The enhancement of enzyme activity in zinc-deficient material was also observed by Nason *et al.* (8), who found that the activity of some enzymes increased as a result of omitting certain micronutrients.

Since it was not possible to determine the formation of tryptophan quantitatively, the possibility that the pathway of indole metabolism is altered in zinc-deficient plants is not eliminated. The conversion of indole to products other than tryptophan would, in fact, account for a simultaneous increase in indole metabolism, and a decrease in the amount of tryptophan present in zinc-deficient plant tissues.

Acknowledgments

The authors wish to express their thanks to the National Research Council of Canada for financial assistance in the form of a Studentship to J. B. Mudd, and for their continued financial support; and to Mr. Mike Ostafichuk for his assistance in photographic and other technical procedures.

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THE INTERACTION BETWEEN DISODIUM ETHYLENEBISDITHIOCARBAMATE (NABAM) AND MANGANESE[1]

R. A. Ludwig and G. D. Thorn

Abstract

The investigations reported in this paper show that trace amounts of manganese catalyze the oxidation of nabam. Conversion to ethylene thiuram monosulphide was found to be pH dependent. The results obtained help to explain the apparently anomalous behavior of nabam in earlier oxidation studies.

Introduction

Nabam and its metal salts can be converted to ethylenethiuram monosulphide (monomer and polymer) by air oxidation (2, 3). This substance possesses the chemical, physical, and biological properties necessary to explain the activity of the parent materials as foliar fungicides. The original work from this laboratory on the conversion of nabam to ethylenethiuram monosulphide has presented certain anomalies. *Firstly:* conversion by air oxidation could only be accomplished with dilute (spray strength or less) nabam solutions. *Secondly:* even with very vigorous aeration conversion was a slow process requiring from 1 to 4 days. *Thirdly:* commercial nabam was used, since attempts to convert pure nabam either failed or, at the best, gave very erratic results. *Fourthly:* certain samples of commercial nabam could not be converted. *Fifthly:* yields of ethylenethiuram monosulphide (ETM) increased in repeated runs as the reaction vessel became coated.

A possible explanation for these effects was suggested by an Australian patent application (4), which claimed high yields of ETM by oxidation of concentrated nabam solutions with oxygen under pressure when traces of manganese salts were added. The present paper constitutes a re-examination of the oxidation of nabam in the light of the Australian report.

The Stability of Nabam Solutions at Various pH Levels

Studies of any of the reactions of the dithiocarbamates must take into account their decomposition under acid conditions. Although this decomposition is well known, little information is available on the rate with which it occurs and hence a study of it was made as a preliminary to the experiments reported in the ensuing sections of this paper. These experiments were carried out in a nitrogen atmosphere in order to protect the dilute nabam solutions from oxidative change. Solutions of chemically pure nabam (0.0025 *M*) were held at the desired pH levels by the addition of dilute hydrochloric acid (either 0.05 or 0.01 *M*) with an automatic titrator.[2] The nabam concentration was determined spectrophotometrically at appropriate time intervals.

[1]Manuscript received March 26, 1958.
Contribution No. 122 from the Science Service Laboratory, University Sub P.O., London, Ontario.

[2]Radiometer titrator type TTT1. Radiometer, Copenhagen, Denmark.

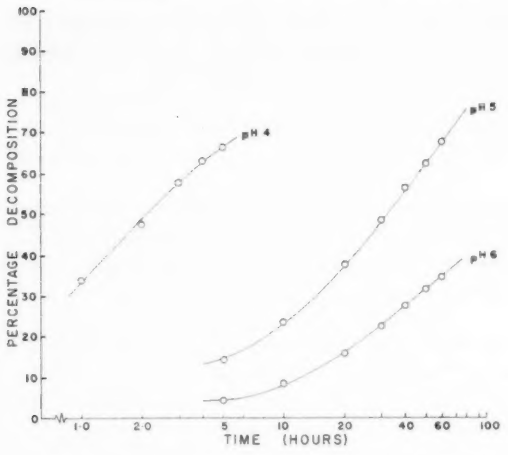


FIG. 1. The stability of nabam at various pH's.

The results, plotted as percentage decomposition against time, are presented in Fig. 1. It will be noted that the half life of nabam is approximately 2 and 30 hours at pH 4 and 5 respectively but is longer than 4 days at pH 6.

The Effect of Manganese Level on Nabam Oxidation

In a series of experiments nabam solutions containing various amounts of manganese were oxidized with oxygen at 10 lb. pressure. One hundred milliliter quantities of solutions, with final 2% concentrations of chemically pure nabam ($C_4H_6N_2S_4Na_2.6H_2O$), contained in 1000 ml. round-bottom flasks, were used throughout. During the course of the reaction the flasks were agitated vigorously with a wrist-action shaker. Manganese sulphate was used since preliminary experiments showed it to be as effective an oxidation catalyst as manganese dioxide.

The oxidation proceeded rapidly, except in the case of manganese-free solutions, and the contents of the flasks were analyzed after 3 hours although the reactions had apparently reached an end point in half an hour.

The concentrations of manganese used and the results obtained in a typical experiment are given in Fig. 2. It will be noted from Fig. 2 that the yield of solid increased with increasing manganese up to the equivalence point. At this point the theoretical yield of manganese ethylenebisdithiocarbamate (maneb) is approximately 1.5 g. If complete oxidative conversion of the maneb took place, approximately 1.0 g. of ethylenethiuram monosulphide and 0.2 g. of sulphur would be formed.

The precipitates were chloroform extracted. The chloroform-insoluble fraction presumably gives a measure of maneb and polymeric ETM as opposed to monomeric ETM and sulphur. The amount of ETM in the chloroform-soluble portion was then determined spectrophotometrically. The precipitates

from low-manganese systems (Fig. 2) were highly soluble and contained a large proportion of ETM. Precipitates obtained in the presence of an equivalence of manganese were virtually chloroform insoluble. The biological activities of the solids, as measured by the spore-drop-dilution technique with *Monilinia fructicola* as the test organism (1), were higher with something less than an equivalence of manganese than they were when equimolar amounts were used. A gradient of activity corresponding to the manganese gradient was not, however, obtained.

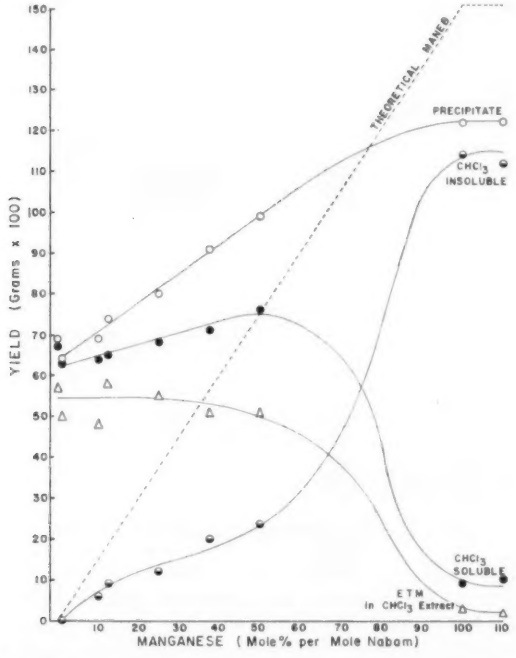


FIG. 2. The oxidation of nabam in the presence of varying amounts of manganese.

The biological activities and apparent ETM contents of the filtrates did not vary greatly over the range of manganese concentration used. The exactness of the ETM values, determined on the filtrates by ultraviolet absorption, is open to question, however, owing to the presence of an absorption peak at 2730 Å which interferes with the 2800 Å peak of ETM. The unidentified substance, in all likelihood di- or tri-thiocarbonate, responsible for this peak remains in the aqueous phase on chloroform extraction as illustrated by the spectrum presented in Fig. 3. Here the spectra of nabam, ETM, and ethylene thiourea (ETU) are also included for purposes of comparison.

The pH of the filtrate when an equivalence of manganese had been used was 6.9. At lower manganese levels it decreased from 11.6 with 1% manganese to 8.7 with 50% manganese.

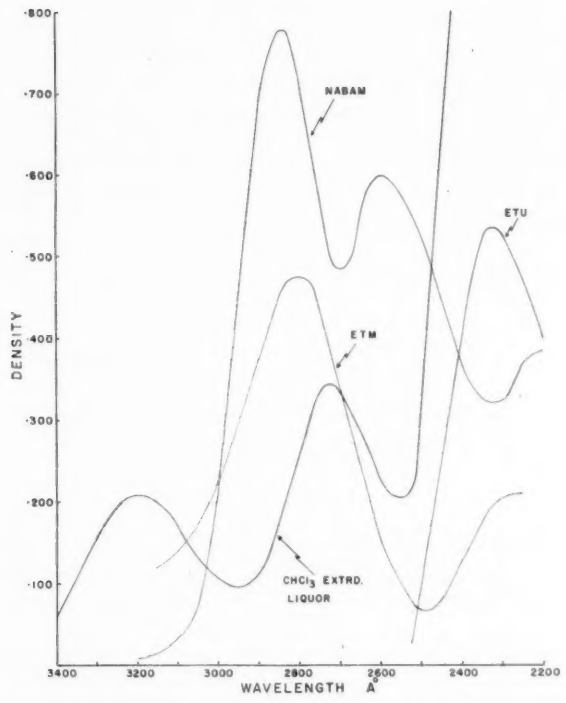


FIG. 3. Ultraviolet absorption spectra of nabam and its oxidation products. Nabam: 2.8×10^{-5} *M*; ETM: 2.4×10^{-5} *M*; ETU: 3.25×10^{-5} *M*.

pH Change During Nabam Oxidation

Aqueous solutions of pure nabam have a pH of about 7.0. This, however, is seldom observed because oxidative decomposition accompanied by an increase in alkalinity readily occurs. This change is illustrated in the pH's found in the filtrates of the preceding experiment. Since the round-bottom flasks used did not permit measurement and control of pH, a special reaction vessel was designed to overcome this difficulty during the pressure oxidation of relatively small volumes of solution. This vessel is illustrated in Fig. 4. During a run the pressure was maintained at 10 p.s.i. and sufficient gas allowed to escape through the pressure-release arm to give rapid circulation of the liquid. All parts of the system (burette, KCl electrode reservoir, etc.) were pressurized to the same degree as the reaction vessel. The automatic titrator previously mentioned was again employed and pH changes were registered on an automatic recorder. In the type of vessel illustrated gas bubbles did not interfere with the operation of the electrodes.

Two grams of nabam was used in each run and 1/100 of an equivalence of manganese provided as manganese sulphate when required. In practice 150 ml. of water with or without manganese was placed in the reaction vessel and

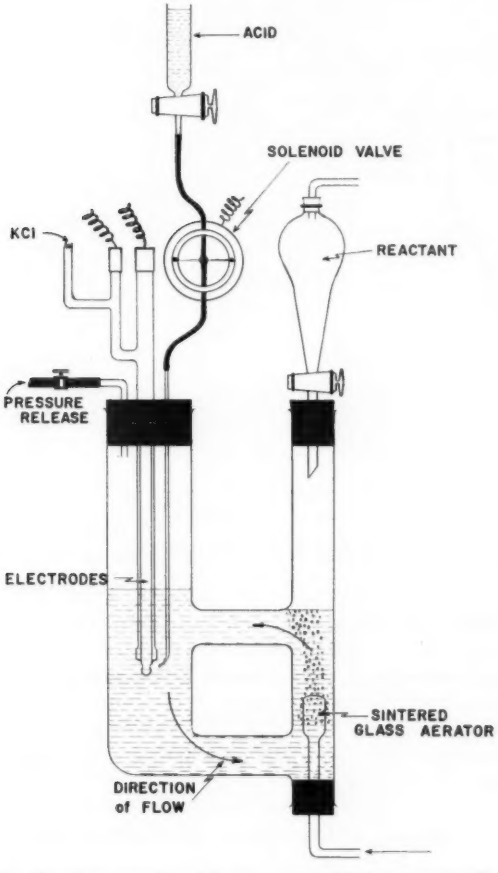


FIG. 4. Reaction vessel used for pressure oxidation at controlled pH.

the separatory funnel charged with the nabam dissolved in 25 ml. of oxygen-free water. The pressure and circulation were then adjusted with nitrogen. The nabam was discharged into the reaction vessel as soon as the oxygen and carbon dioxide had been driven from the system (pH 7). At time 0 of Fig. 5 the nitrogen was replaced with air or oxygen.

The pH under nitrogen reached a constant level of 9.7 to 10.0. This high initial pH indicates that the "chemically pure" nabam used had already undergone some oxidative change. The striking feature of the curves in Fig. 5 is the rapid rise of pH in the presence of manganese. A peak of approximately pH 12 was achieved in 30 to 40 minutes, at which point precipitation was complete and the pH began to drop. The curves for solutions lacking manganese present a quite different picture. Here no precipitation occurred in

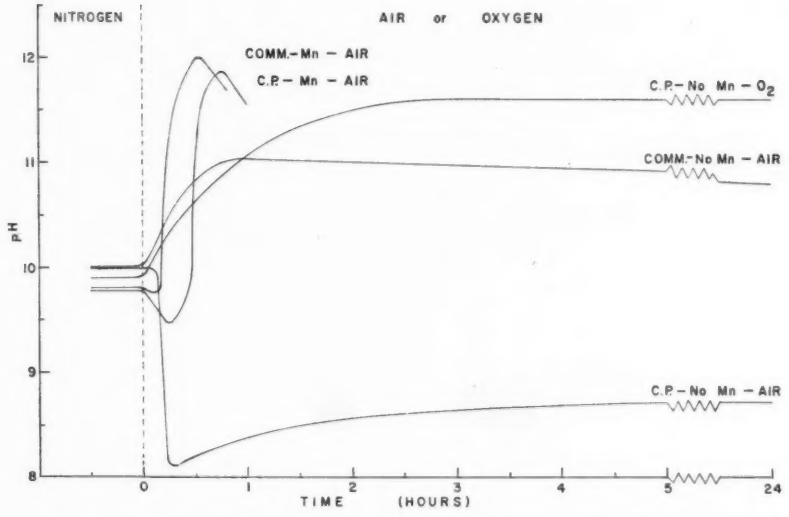


FIG. 5. pH changes occurring during the oxidation of commercial and chemically pure nabam solutions.

24 hours although some change had obviously taken place, since the solutions had turned a deep orange and a gradual rise in pH had occurred. Further, the solutions had achieved some sort of equilibrium as indicated by the flattening of the curves after the first few hours. The initial drop in pH with air as the oxidant may be due to acidification with carbon dioxide. The difference in the curves obtained with chemically pure nabam under oxygen and air supports this view.

Nabam Oxidation at Controlled pH

A study was made of the oxidation of nabam with and without manganese at various controlled pH's. The procedure was essentially the same as that used in the preceding section, 2 g. of nabam (chemically pure) and a 0.01 *M* equivalence of manganese as manganese sulphate again being used. The pH was controlled by the addition of 0.2 *M* HCl. Oxygen at a pressure of 10 p.s.i. was used throughout.

Fig. 6 shows the rate of acid addition with and without manganese at various pH's. Approximately 55 ml. of HCl would be required to neutralize the sodium ions released from the nabam. Twice this amount would be consumed if complete breakdown to ethylene diamine and carbon disulphide took place.

After an appropriate period of time the reaction was stopped and the solids separated from the liquid by filtration. The filtrate was then examined spectrophotometrically for residual nabam and dissolved ETM and ETU.

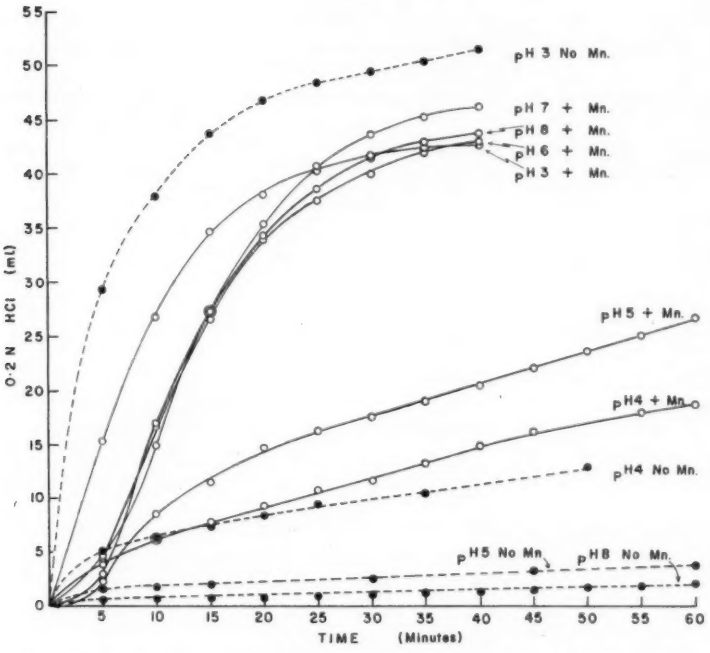


FIG. 6. Acid consumption during the oxidation of nabam at controlled pH's.

The results are given in Table I. It will be noted that ETM appeared only at pH's 6, 7, and 8 and then only in the presence of manganese. In these cases the nabam had completely disappeared. The other solutions, including pH 8 without manganese, still contained nabam.

TABLE I

ETM AND RESIDUAL NABAM IN SOLUTIONS OXIDIZED AT VARIOUS pH's

pH	Mn^{++}	Sampling time (min.)	Residual nabam (grams)	ETM (grams)
3	+	40	0.69	—
	−	40	0.33	—
4	+	110	0.34	—
	−	not done		
5	+	240	0.39	—
	−	90	1.37	—
6	+	40	—	0.077
	−	not done		
7	+	40	—	0.113
	−	not done		
8	+	40	—	0.075
	−	165	1.09	—

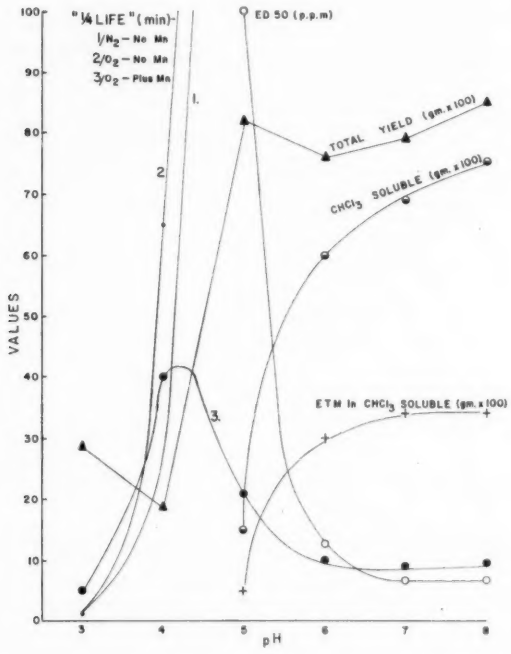


FIG. 7. The effect of pH on the oxidation of nabam. For a detailed explanation see text.

Further details of the reactions are given in Fig. 7. Here an attempt has been made to compare rates of acid consumption with (curve 3) and without (curve 2) manganese at various pH levels by plotting the time required to one-quarter neutralize the two sodium ions of nabam. This rather unorthodox statistic ("$\frac{1}{4}$ life" of Fig. 7) was selected because many of the reactions were not carried on for a sufficient length of time to get a complete or even 50% neutralization point. For purposes of comparison the "$\frac{1}{4}$ life" of nabam under nitrogen (curve 1) derived from Fig. 1 has also been included. The influence of acid decomposition on the course of the reaction at low pH's is obvious. This effect is negligible at pH's of 5 and above. The curve for solid residue is based on reactions with manganese. It will be noted that some precipitation occurred at pH's 3 and 4. These residues were very unstable and were in all likelihood ethylenebisdithiocarbamic acid. Residues at pH's 5, 6, 7, and 8 were approximately equal in weight. The one at pH 5, however, was much less chloroform soluble and had a lower ETM content and biological activity than the other three.

The nitrogen and sulphur balances for the manganese-containing systems at pH's 6, 7, and 8 are given in Table II. The approximate N:S ratio of 1:2

TABLE II

NITROGEN AND SULPHUR BALANCE IN CATALYTICALLY OXIDIZED NABAM SOLUTIONS AT VARIOUS pH's*

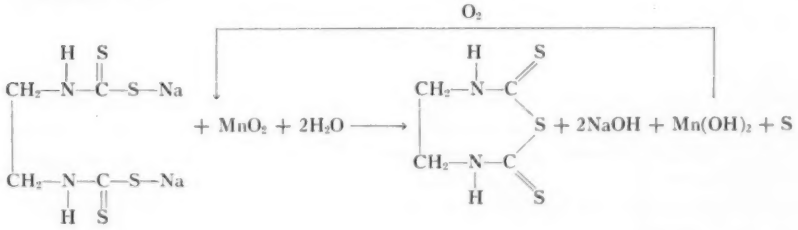
pH	ETU† in filtrate	Theor. CS_2‡	ETM§ in filtrate	Residue	Total	Expected
			Nitrogen			
6	3.6		0.9	7.2	11.7	11.0
7	3.4		1.3	7.2	11.8	11.0
8	2.4		0.9	7.2	10.5	11.0
			Sulphur			
6	1.8	3.6	1.3	14.7	21.4	22.0
7	1.6	3.2	1.8	15.1	21.7	22.0
8	1.2	2.4	1.2	15.4	20.2	22.0

*Figures are expressions of miliatoms N or S in the reaction components.
†Ethylenethiourea.
‡Assuming 1 M. CS_2 formed per mole of ETU found (2).
§Ethylenethiuram monosulphide.

found in the residues is that expected for either ethylenethiuram disulphide of a 1:1::ETM:sulphur mixture. These were not resolved but the curves of Fig. 7 suggest that the residues were mixtures of all three.

Discussion

The mechanisms of the reaction between nabam and a metal such as manganese are not easy to resolve. One explanation of the over-all reaction involved in the catalytic effect of manganese on oxidation is embodied in the following equation:



Manganic oxide and manganous sulphate were equally effective in promoting the reaction. Color changes suggested that the latter was rapidly converted to the former under the conditions of pH and oxygen tension prevailing in the experiments reported here. Such factors as the oxidation–reduction potentials, strength of the co-ordination complex, and the solubility of the reaction products will be involved in the rate and course of the reaction.

The interaction between nabam and traces of metals such as manganese furnishes at least a partial answer to the questions raised in the *Introduction*

since traces of various metals were obviously present in earlier reaction systems. Many details of the reaction are, however, still obscure and work aimed at their elucidation is continuing.

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GLUCOSE CATABOLISM IN PEPPER FRUIT (CAPSICUM FRUTESCENS LONGUM)[1]

W. P. Doyle and C. H. Wang

Abstract

The glucose catabolism in several varieties of pepper fruit has been investigated by means of tracer techniques employing glucose-1, -2, -3(4), and -6-C^{14} as the labelled substrates. A method is presented whereby the participation of individual pathways in these fruits may be identified and estimated. It was found that 28–36% (among the varieties tested) of the glucose catabolized was routed through a direct oxidative pathway, the remainder being oxidized via the Embden–Meyerhof–Parnas and tricarboxylic acid cyclic processes. No significant difference in pathway distribution was found between green mature peppers and red mature peppers.

Parallel to the elucidation of the path of carbon in photosynthetic processes, the catabolism of glucose in plant materials has also been the subject of many recent studies (1, 3, 4, 6). Radioactive-tracer techniques were employed by Gibbs *et al.* (3) in demonstrating the occurrence of the direct oxidative pathway in plant tissues; however, the quantitative evaluation of various catabolic pathways of glucose in intact fruit is yet to be thoroughly investigated. This is primarily due to the difficulties encountered in administering a C^{14}-labelled substrate uniformly into an intact plant system and in estimating the exact amount of the labelled substrate engaged in catabolic functions during a given period.

In the present work, using fruits of the *Capsicum* genus as test systems, a method has been devised to identify and estimate the catabolic pathways of glucose in this fruit employing tracer techniques.

The findings indicate that the bulk of the fruit glucose was degraded by way of a sequential route consisting of the Embden–Meyerhof–Parnas (EMP) glycolytic pathway and the tricarboxylic cycle (TCA).

Method

Fruits

All fruits used in the present experiment were obtained from the local markets, except for experiment 3 in which fruits were supplied through the courtesy of Mr. John Keene. Variety identification was established through the kind co-operation of Professor W. A. Frazier. The fruits were carefully selected to insure reasonable uniformity with respect to their physiological state.

Radioactive Substrates

The C^{14}-labelled substrates used in these experiments were acetate-1-C^{14} and acetate-2-C^{14} obtained from Tracerlab; glucose-1, -2, and -6-C^{14} from the

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Contribution from the Department of Chemistry, Oregon State College, Corvallis, Oregon. This research was supported in part by contract (AT-(45-1)-573) from the Atomic Energy Commission, published with the approval of the Monograph Publication Committee, Research paper No. 329, School of Science, Department of Chemistry.

National Bureau of Standards through the co-operation of Dr. Isbell; and glucose-3(4)-C^{14} prepared in this laboratory according to the method of Wood *et al.* (8).

Administration of Substrate

The labelled substrate was introduced into the fruit by means of a technique devised for *Capsicum* fruit using the principle of vacuum infiltration. The operation was carried out in a respiration chamber shown in Fig. 1. Into the substrate well (E) was delivered 0.2 cc. of an aqueous solution containing 0.5 μc. of the specifically labelled substrate having a preadjusted specific activity of 1.0 μc/1.0 mg. The test fruit was lowered into the chamber in such a way that the freshly cut stem of the fruit extended to the bottom of the well. A partial vacuum was then applied to the chamber to allow air inside the fruit to escape through the stem as a slow stream of bubbles.

Upon partial restoration of the pressure in the chamber, the solution in the well rapidly infiltrated the vascular tissue of the fruit through the stem opening. Then 0.4 cc. of distilled water was introduced to the substrate well through the stopcock side arm (D) and was subsequently taken up by the fruit when atmospheric pressure was fully restored in the respiration chamber.

It was observed by preliminary experiments that this method of substrate administration results in uniform distribution of the labelled compound throughout the vascular tissue. The radiochemical recovery of substrate activity in respiratory CO_2 among fruit treated in a similar manner was reproducible within 5% of the total recovery.

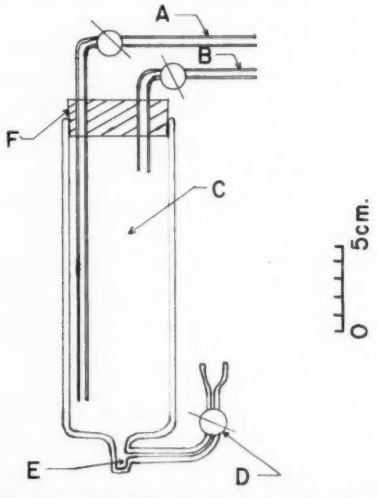

FIG. 1. Scale drawing of a typical flask used for infiltration of substrate and subsequent sweeping with CO_2-free air for the collection of respired CO_2. (A) Air outlet from which the air goes directly to CO_2 trap; (B) air inlet tube; (C) respiration chamber; (D) capillary stopcock for admitting wash water; (E) substrate well; (F) two-hole rubber stopper.

Time-course Experiments

The time course of the radiochemical recovery of the substrate activity in respiratory CO_2 from fruit utilizing C^{14} specifically labelled compounds was followed in a manner similar to that described previously for microbial studies (7). Essentially, each respiratory chamber is connected to an air supply and a master flowmeter by means of a bypass manifold. The metabolic CO_2 in the chamber was swept into a CO_2 trap which consists of a sintered-glass sparger immersed in 20 cc. of 0.5 *N* CO_2-free NaOH. The trap solution was periodically replaced and processed for recovery of carbonate as $BaCO_3$.

Radioactive Measurement

$BaC^{14}O_3$ was mounted on aluminum planchets using centrifugation techniques and was counted with a GM counter to a standard deviation of 2%. All counting data were corrected for background and self-adsorption in the conventional manner.

Results and Discussion

Inasmuch as acetate is probably one of the most important key intermediates in the catabolism of glucose in biological systems, specifically C^{14}-labelled acetates were used as the first test substrates in the present study. The preferential conversion of C-1 of acetate to CO_2 as compared to that of C-2 as shown in Fig. 2 is in line with the operation of the TCA cycle in this fruit. The data thus render support to the recent work of Howard and Yamaguchi (5) in which the oxidation of TCA intermediates by the particulates isolated from pepper were observed.

In the case of glucose catabolism two main pathways have been reported in the plant kingdom, namely, the EMP glycolytic process and the direct oxidative route, the latter being closely related to the photosynthetic mechanism. In the present work much information on the nature of the catabolic pathways can be gained by examining the interval radiochemical recovery in CO_2 collected from peppers metabolizing glucoses -1, -2, -3(4), or -6-C^{14} respectively. Thus in Fig. 3 it is noted that the conversion of C-3(4) of glucose to CO_2 is prompt and profound evidently as a result of the decarboxylation of pyruvate derived from glucose via the EMP pathway. The acetate formed in this process, having its methyl carbon equivalent to C-1 or C-6 and its carboxyl carbon equivalent to C-2 and C-5 of the glucose molecule, is presumably metabolized by way of the TCA cyclic processes, which should then result in a more rapid conversion to CO_2 of C-2 as compared to C-1 and C-6. The fact that the direct oxidative pathway is also operative in this fruit is evidenced by the observed preferential combusion of C-1 to CO_2 in contrast to the much more reluctant appearance of C-6 and C-2 of glucose in CO_2 as shown in Fig. 3. It is conceivable that the phosphogluconate decarboxylation may account for the latter observation. There also remained a possibility that a portion of C-2 of glucose may have been combusted by way of the pentose-cycle reactions, although the extent of the interaction of this type

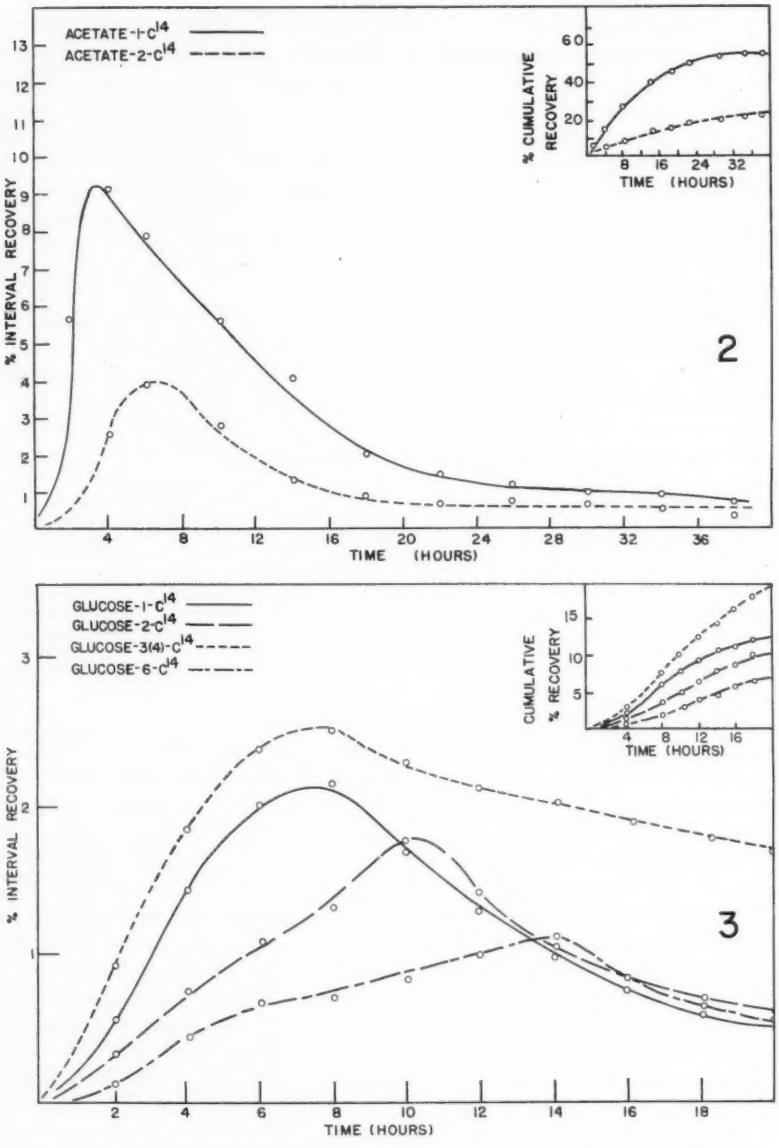

FIG. 2. Cumulative and interval (%) radiochemical recovery of C^{14} in expired CO_2 from fruits utilizing acetate-1 or -2-C^{14}.

FIG. 3. Cumulative and interval (%) radiochemical recovery of C^{14} in expired CO_2 glucose-1, -2, -3(4), or -6-C^{14}.

cannot be defined in the present experiment. Similarly, C-6 of glucose could have been converted to CO_2 by way of a sequence of reactions involving triose isomerase, reverse aldolase, and a 1–5 cleavage of the resulting hexose. However, in view of the delayed appearance of the maximum in the time-course plot of the interval radiochemical recovery of $C^{14}O_2$ from glucose-6-C^{14}, it is doubtful that the operation of this sequence, if occurring, is extensive. In order to estimate the quantitative contribution of individual pathways to the over-all catabolism of glucose in this fruit it is necessary to determine first the amount of administered C^{14}-glucose actually engaged in catabolic processes. It can be visualized that the labelled glucose, once introduced to the fruit system, is subjected to a variety of destinations. Thus, the labelled glucose is gradually diluted by the unlabelled glucose in the metabolic pool resulting in a continuous decline of the specific activity, while a portion of the labelled glucose will be degraded in the catabolic functions with the remainder converted to various polysaccharides. The combined consequences of these processes is indicated by the rapid increase of the rate of $C^{14}O_2$ recovery from glucose-C^{14} in the early phase and the subsequent decline as shown in Fig. 3. Since a reasonably homogeneous system with respect to substrate distribution is realized in the present experiment, the relative rate of $C^{14}O_2$ recovery from glucose labelled in the different positions should then reflect directly the catabolic pathways followed by the respective carbon atoms.

In view of the experimental difficulties involved in determining directly the exact amount of labelled glucose engaged in catabolic functions, in the present work the latter is estimated according to equation (1).

(1) $$G_t = (G_1 - G_6) + G_{3(4)}$$

where

G_t = percentage of the labelled glucose engaged in catabolic functions during time period t.

G_1 = percentage cumulative recovery in CO_2 from glucose-1-C^{14} during time period t.

G_6 = percentage cumulative recovery in CO_2 from glucose-6-C^{14} during time period t.

$G_{3(4)}$ = percentage cumulative recovery from glucose-3(4)-C^{14} in CO_2 during time period t.

The above equation is derived from the fact that for each mole of glucose catabolized there should result 1 M. of CO_2 from C-1 of glucose via the direct oxidative route (i.e. $G_1 - G_6$, since C-1 and C-6 behave identically in the EMP pathway (7)) and 1 M. of CO_2 should be derived from either C-3 or C-4 by way of the EMP glycolytic pathway.

It is assumed, evidently, that the decarboxylation of C-1 or C-3(4) by the respective catabolic pathway is prompt and quantitative. This implies that the C_3 compounds such as pyruvate are not accumulating or involved in direct biosynthetic functions. The finding that the incorporation of glucose into the acid and amino-acid fractions of tomato fruit is relatively insignificant (2) tends to support the validity of the above assumption. It is also understood

that the utilization of pentose (as derived from glucose) by way of any degradative reactions will result in conversion of C-3(4) and C-6 to CO_2 in a manner other than the EMP glycolytic pathway. However, it is felt that the interference from this consideration, which is secondary in nature, would not inflict any significant effect on the use of equation (1) when the latter is applied to the data collected in the early phase of the time-course experiment.

In the present work the estimation of the catabolic pathways of glucose is carried out by taking into consideration the following assumptions; these assumptions are derived from the available information of the reaction rates and the nature of catabolic reactions of glucose in plants and microorganisms.

1. Glucose is metabolized degradatively via only two pathways: EMP coupled with the TCA cycle and a direct oxidative route involving the preferential decarboxylation of C-1.
2. The pyruvate formed in glycolysis is promptly decarboxylated.
3. The phosphogluconate decarboxylation is a rapid reaction.
4. The pentose formed from the phosphogluconate decarboxylation is only slightly metabolized in the early phase of the experiment by way of either the pentose cycle or degradation to C_2 and C_3 intermediates.
5. The trioses formed in glycolysis are equivalent to each other in further catabolic processes.
6. The recombination of labelled trioses to form a hexose is not extensive.

The percentage of the glucose catabolized by the direct oxidative pathway, designated as G_p, can then be calculated according to equation (2).

$$G_p = (G_1 - G_6)/G_t \times 100 \tag{2}$$

and the percentage of the glucose catabolized via the EMP–TCA pathway is:

$$G_e = 100 - G_p \tag{3}$$

In Table I is given the time course of the cumulative radiochemical recovery in CO_2 from pepper (var. chilli) metabolizing glucose-1, -2, -3(4), or -6-C^{14}, along with the values of G_t, G_p, and G_e calculated according to equations (1), (2), and (3). It will be noted in Table I that the value of G_t increases with

TABLE I

THE FATE OF CARBON ATOMS OF GLUCOSE IN PEPPER (*Capsicum frutescens* LONGUM)

Time (t) in hours	% Cumulative radiochemical recovery in CO_2 from glucose				G_t (Eq. 1)	G_e (Eq. 3)	G_p (Eq. 2)
	-1-C^{14}	-2-C^{14}	-3(4)-C^{14}	-6-C^{14}			
2	0.49	0.41	0.94	0.10	1.33	70.7	29.3
4	1.9	1.2	2.7	0.6	4.00	67.5	32.5
6	3.95	2.2	5.0	1.3	7.65	65.4	34.6
8	6.3	3.5	7.5	2.0	11.8	63.6	36.4
10	8.0	5.3	9.7	2.8	14.9	65.1	34.9
12	9.4	6.5	11.8	3.8	17.4	67.8	32.2
14	10.4	7.5	14.0	4.9	19.5	71.8	28.2
18	11.8	9.4	17.9	6.2	23.5	76.2	23.8
22	13.0	11.1	20.7	7.4	26.3	78.7	21.3
26	14.0	12.7	22.8	8.6	28.2	80.9	19.1
30	15.0	14.1	24.9	9.7	30.2	82.5	17.5
34	15.8	15.7	26.5	10.5	31.8	83.3	16.7

time at an approximately constant rate in the early phase (from 0 to 8 hours) and tapers off toward the end of the experiment. Although the calculations could be made on the data collected at any given time in the early phase according to equations (2) and (3), it appears advisable, in view of the previous discussion, to carry out the calculation at that time when $C^{14}O_2$ from C-1 of glucose registers a maximum rate of recovery (8 hours, Fig. 3) when applying the present method to fruit in general.

In this particular variety of pepper (as shown in Table I), 36% of the glucose entering catabolic processes was degraded by way of the direct oxidative pathway, with the remaining fraction being degraded by the EMP–TCA route. This implies that a maximum amount of 36% of the catabolized glucose was converted to pentose.

In order to detect the possible variation with respect to pathway distribution among various horticultural varieties of pepper fruit, similar experiments have been carried out with three other varieties available at the time. The cumulative radiochemical recovery in CO_2 from labelled glucose in these fruits together with the calculated values of G_t, G_p, and G_e are given in Table II. In the present case the latter values are given at a time chosen for calculation as discussed previously. It thus appears that the participation of individual pathways does not vary to a great extent among the varieties of peppers examined.

TABLE II

PATHWAY PARTICIPATION OF GLUCOSE CATABOLISM IN FOUR VARIETIES OF PEPPER (*Capsicum frutescens* LONGUM)

Expt. No.	Horticultural variety	% Cumulative radiochemical recovery in CO_2 from glucose*				G_t (Eq. 1)	G_e (Eq. 3)	G_p (Eq. 2)
		-1-C^{14}	-2-C^{14}	-3(4)-C^{14}	-6-C^{14}			
1	Chilli	6.3	3.5	7.5	2.0	11.8	64	36
2	Yellow wax	5.5		10.2	1.6	14.1	72	28
3	Not determined	11.9		18.4	4.9	25.4	72	28
4	Cayenne (green mature)	15.9		16.7	5.9	26.7	64	36
5	Cayenne (red mature)	10.2		12.6	3.9	18.9	67	33

*These values constitute the averages of two experiments.

Experiments 5 and 6 in Table II were designed to determine if the development of red pigment is accompanied by a variation in pathway participation. The data in Table II indicate that there was no significant difference in this respect; however, an over-all decrease in recovery was observed as indicated by the values of G_t. This finding could be the result of either a decline of enzyme activity or a greater dilution of the labelled substrate in the red mature fruit. The first possibility was ruled out on the basis of equal respiration rates. The second possibility thus appears to be a probable explanation in view of the larger amounts of free sugar in ripe fruits.

The authors are grateful to Professor E. Hansen for his kind co-operation in this work.

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DECAY OF TREMBLING ASPEN[1]

J. T. BASHAM[2]

Abstract

A total of 1754 aspen trees were examined on 47 sample plots located in even-aged stands considered representative of the study area. Decay was found in the merchantable portions of the trunks of 1212 or 69% of these trees. Three types of heart rot were encountered, viz., a white spongy trunk or butt rot and a yellow stringy trunk rot associated respectively with *Fomes igniarius* var. *populinus* (Neu.) Campb., and *Radulum casearium* (Morgan) Lloyd; and stringy butt rots from which several fungi, notably *Pholiota spectabilis* Fr. and *Armillaria mellea* (Vahl ex Fr.) Quél., were isolated. Two heartwood stains, one brown and the other mottled red, were present in many of the aspen trees examined. *Corticium polygonium* Pers. and two members of the Fungi Imperfecti, *Libertella* sp. and *Phialophora alba* van Beyma, were consistently isolated from these stains and from incipient decays. Fructifications of *F. igniarius* were the most reliable external indication of heart rot in this species. A pronounced relationship existed between age and decay. Older stands as a rule contained higher proportions of decayed heartwood than young stands. A less pronounced relationship was found to exist between soil conditions and decay; trees occurring on drier sites were somewhat more decadent than those growing on moist sites. The possible reasons why the decay process in the heartwood of living aspen is one in which certain fungi, mainly Fungi Imperfecti, colonize and stain the wood prior to its invasion and destruction by the Basidiomycetes associated with advanced decay are discussed.

Introduction

Trembling aspen, *Populus tremuloides* Michx., frequently called aspen, aspen poplar, or poplar, extends from the Atlantic Provinces to the west coast of British Columbia, and to the Yukon in the northwest. The name aspen is used for this species throughout this paper. In northern Ontario it accounts for between 15 and 30% of the primary growing stock on productive forest lands.[3] Since World War II, new techniques for handling and processing aspen have been developed by the pulp and paper industry, and the volume manufactured annually into pulp in northern Ontario has increased considerably.

The relative susceptibility of aspen stands to heartwood decay has hindered the utilization of this species. Marginal stands in which the recovery of sound wood would have proved profitable are often rejected, whereas in other stands operations are undertaken which, because of excessive heart rot, are highly unprofitable. When requests were received from the pulp and paper industry, studies were begun to determine the age at which poplar stands should be cut to avoid serious losses and whether the nature of the site significantly affects the rate of decay of these stands.

Fomes igniarius has been recognized since 1909 as one of the major causes of decay of aspen in North America (10). Very little information has been published concerning other fungi associated with heartwood stains and decays

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in this species. These studies provided an excellent opportunity to collect information on the occurrence and relative importance of many other fungi associated with the pathology of living aspen heartwood.

Location and Description of Stands Sampled

This study was carried out in northern Ontario in what is referred to as the Upper Pic region, roughly 150 miles east of Lake Nipigon. The growing season in this region is quite short. The annual precipitation is only about 24 in., but with the relatively brief evaporating season the region has adequate moisture to support forest growth.

The geology and topography of the Upper Pic region have been profoundly affected by glaciation. The slopes and hilltops generally support stands of aspen with some white birch (*Betula papyrifera* Marsh.). Spruce and balsam fir (*Abies balsamea* (L.) Mill.) are usually scattered throughout these stands; the latter is particularly noticeable where the broad-leaved species are overmature. Black spruce (*Picea mariana* (Mill.) B.S.P.), white spruce (*Picea glauca* (Moench) Voss.), and balsam fir are found on the lower slopes and in the valleys, while jack pine stands occur on many of the sandy outwashed plains.

Forty-seven plots, ranging in size from $\frac{1}{10}$ to $\frac{1}{2}$ ac., were established in pure and mixed aspen stands of different ages and on different sites. These stands were considered to be representative of aspen in the Upper Pic region. Aspen accounted for over 60% of the trees of merchantable pulpwood size located on the 10 acres sampled by means of these plots; balsam fir, black spruce, white spruce, and white birch accounted for most of the remainder. Balsam fir accounted for 70% of the trees that were under merchantable pulpwood size. The underbrush of these stands was composed mainly of mountain maple, witch hazel, mountain ash, and alder.

Method of Study

On each plot all living aspen trees measuring over 5 in. in outside bark diameter at a height of $4\frac{1}{2}$ ft. were felled and bucked into 4- or 8-ft. bolts up to a $3\frac{1}{2}$-in. top diameter. Each bolt was carefully examined for stain and decay, and, when necessary, bolts were further sectioned to determine the extent of disappearing decays. Each tree was plotted and described as thoroughly as possible on logarithmic tree measurement forms. The total volume, total

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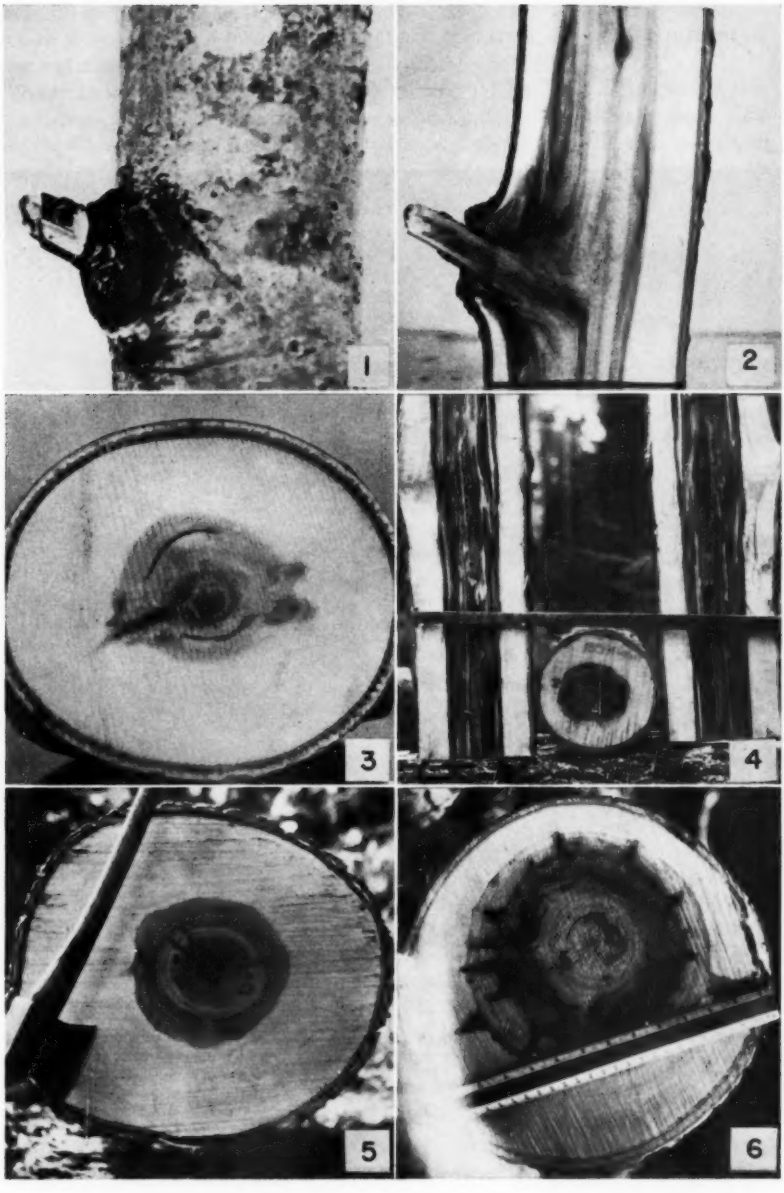
FIG. 1. Branch stub which has served as the point of entry for *Corticium polygonium*.
FIG. 2. Longitudinal section through the branch stub illustrated in Fig. 1, showing the development of *C. polygonium* stain and incipient decay from this locus.
FIG. 3. Transverse section of aspen with light-brown stain and incipient decay caused by *C. polygonium*.
FIG. 4. Longitudinal and transverse sections of the trunk of a young aspen tree, showing extensive red mottled stain in the heartwood.
FIG. 5. Transverse section of aspen with light-brown stain surrounding small pockets of *Fomes igniarius* decay.
FIG. 6. Transverse section of aspen with red mottled stain surrounding incipient decay.

PLATE I



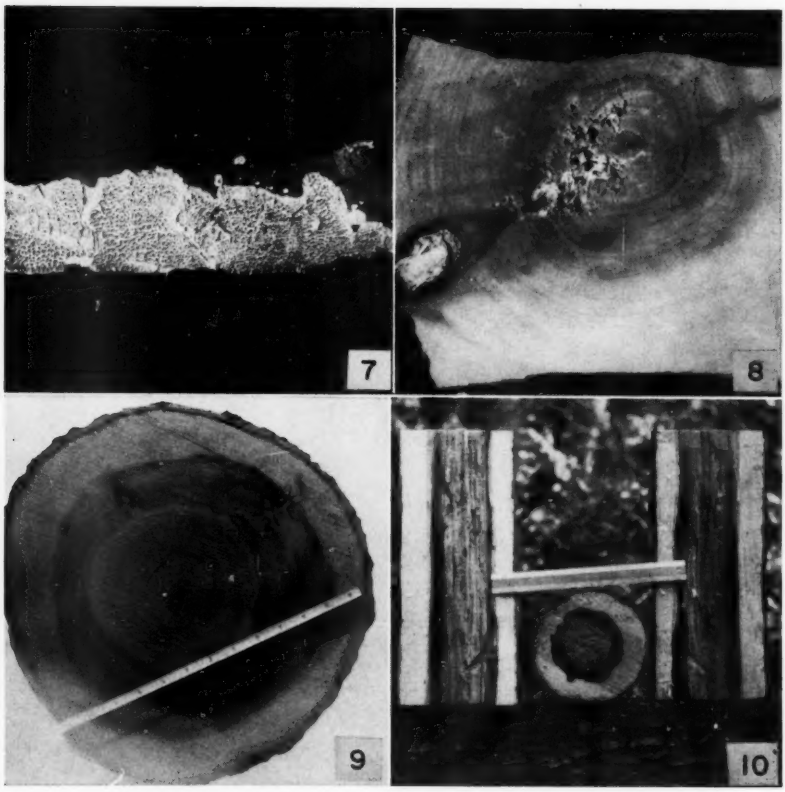


FIG. 7. *Radulum casearium* fructification growing on the underside of an old aspen log.
FIG. 8. Transverse section of aspen with a small central core of *R. casearium* decay, and red stain associated with a knot.
FIG. 9. Transverse section of *R. casearium* decay in aspen.
FIG. 10. Longitudinal and transverse sections of aspen with advanced *R. casearium* decay.

merchantable volume, and volume of decay and stain in the merchantable portion were computed in cubic feet for each tree from the areas representing these volumes on the forms.

Wood that was noticeably softer than normal healthy aspen wood was classified as decay. In many instances, particularly in younger trees, this reduction in strength of decayed wood was very slight; wood in this condition was referred to as incipient decay. Discolored wood which, when tested with the point of a sharp knife, appeared to have qualities of healthy wood was recorded as stain.

Past experience has shown that decay in aspen associated with *Fomes igniarius* var. *populinus* (Neu.) Campb. is quite distinctive and easily recognizable, so that it can be identified in the field by visual inspection. For this reason, only a limited number of samples of this decay were collected. Samples were collected of all other decays and about one-half of the stains encountered; these were shipped to the Maple Laboratory where the fungi associated with these defects were isolated. The organisms which could not be identified at Maple were sent to the Botany and Plant Pathology Division at Ottawa, where they were identified by Dr. M. K. Nobles and her staff.

Stand ages were determined by counting the annual rings of aspen trees at stump height.[4] Each sample plot was assigned to one of four site types, based mainly on an estimate of the availability of soil moisture arrived at by examining soil pits, topography, and other features. All visible wounds and fungus sporophores were recorded for each tree.

Decay and Stain in Living Aspen

A total of 1754 aspen were felled and examined during the course of this investigation. Decay was detected within the merchantable portion[5] of the boles of 1212 or 69.3% of these trees. Butt rot was encountered in 530 or 30.2% of the trees, and 1108 or 63.2% were infected with trunk rot. Practically all the 542 trees with no detectable decay occurred in stands younger than age class 100 years. All trees with the exception of a few in the younger stands had some stained heartwood.

Two distinctly different stains were frequently encountered in aspen heartwood. The first of these was light brown to dark greyish brown (Figs. 2, 3, and 5); the second was predominantly red, although usually mottled in appearance, with yellow, brown, and green hues (Figs. 4 and 6). Both stains occurred extensively in the central heartwood of aspen in young stands prior to or concurrent with the commencement of heart rot. It was sometimes difficult to distinguish between stain and incipient decay, particularly in younger trees.

As aspen matures, portions of the heartwood almost inevitably become decayed. In the aspen sampled, these heart rots were invariably surrounded by stained wood, usually brown. This stain was light brown and generally

[4]Roughly 1 ft. above ground level.
[5]Between stump height and a 3½-in. top diameter.

TABLE I

THE OCCURRENCE OF HEART ROT AND STAIN AT DIFFERENT AGES IN ASPEN IN THE UPPER PIC REGION OF ONTARIO

Age class	Percentage of the total volume of trees stained within the merchantable portions of those trees			Percentage of the total volume of trees decayed within the merchantable portions of those trees			
	Red mottled stain	Brown stain	Total stain	White spongy rot (*Fomes igniarius*)	Stringy trunk rot	Stringy butt rot	Total rot
41– 60	3.2	9.4	12.6	0.7	0.2	0.1	1.0
61– 80	1.5	13.2	14.7	1.4	1.3	0.0	2.7
81–100	1.4	16.7	18.1	3.6	1.0	0.4	5.0
101–120	1.2	17.6	18.8	4.7	3.2	0.3	8.1
121–140	1.9	21.7	23.6	6.1	1.5	0.4	8.0
141–160	2.2	20.4	22.6	12.3	1.2	1.1	14.6
161–180	2.1	22.3	24.4	10.9	1.3	4.6	16.8
Average	1.7	18.2	19.9	6.4	1.5	0.7	8.7
Total volume of defect (cu. ft.)	679.2	7,173.3	7,852.5	2,532.6	605.3	273.5	3,411.4
Percentage of total rot or stain	8.6	91.4		74.6	17.7	8.0	

quite extensive when associated with small or incipient decays, and dark brown to greyish brown in the form of a fairly narrow collar surrounding extensive, advanced decays. Red mottled stain occasionally replaced the brown stain in this association, and was frequently observed mixed with the extensive brown stain which was practically always present at stump height in aspen of all ages. In many mature, and overmature, defective trees this mottled stain was the only interior defect in the smaller top logs cut in the vicinity of the crowns. Red mottled stain was also frequently associated with knots and dead branch stubs in trees of all ages.

The percentages of the total tree volume affected by the two types of stain at different stand ages are presented in the first part of Table I. It is evident that red mottled stain occupied approximately 2% of the total tree volume at all ages, whereas the percentage of the volume affected by brown stain increased from about 10% in stands 41 to 60 years old to over 20% in stands over 120 years old.

The decays encountered in living aspen may be divided into three broad types. The first and most prevalent type is that associated with *Fomes igniarius*. This is a yellowish-white trunk or butt rot with characteristic black zone lines; its texture varies from fairly firm to punky or spongy, depending upon the stage of decay. The second type is a yellow or yellow-brown stringy trunk rot, that is, it occurs in the main part of the bole and seldom reaches ground level. All stages of this decay were encountered, ranging from incipient to advanced (Figs. 2, 3, 8, 9, and 10). The third type is a yellow or yellow-brown stringy butt rot that is confined to the roots and base of the tree. This decay usually occurs in cones of rot 1 to 3 in. in diameter at stump height, tapering out as a rule 2 or 3 ft. above ground level although in a few instances extending to 9 ft. Most of these decays were advanced.

The two types of trunk rot were frequently mingled in a single tree, and both stage of decay and diameter of rot varied considerably at the different saw cuts. In overmature trees they generally occurred as extensive columns throughout most of the merchantable bole, occupying all or most of the heartwood. As mentioned previously, all three types of decay were always surrounded by firm, stained wood. The average volume of decay in the merchantable portion per infected tree was 3.4 cu. ft. for *Fomes igniarius* decay, 1.1 cu. ft. for yellow stringy trunk rot, and 0.6 cu. ft. for stringy butt rot. Although there were almost as many trees infected with yellow stringy trunk rot as with *Fomes igniarius* decay, the latter type of rot accounted for roughly four times as much decay on a volume basis as the former (Table I).

The percentage of the total volume of the trees sampled affected by the three types of decay at different stand ages is presented in part 2 of Table I. In stands over 60 years old, age apparently had very little effect upon the percentage of the total volume affected by yellow stringy trunk rot, whereas there was a marked relationship between age and the percentage of the total volume affected by *Fomes igniarius* decay. Almost three fourths of the total volume of rot encountered in these trees was attributed to *Fomes igniarius*.

TABLE II

FUNGI ISOLATED FROM YELLOW STRINGY TRUNK ROT OF ASPEN AT DIFFERENT AGE CLASSES IN THE UPPER PIC REGION OF ONTARIO

Organism	Number of isolations							
	Age class							
	41–60	61–80	81–100	101–120	121–140	141–160	161–180	Total
Radulum casearium (Morgan) Lloyd	—	4	36	53	59	62	12	226
Corticium polygonium Pers.	6	17	6	7	—	—	—	36
Pholiota adiposa Fr.	1	—	—	1	—	1	—	3
Gloeocystidium karstenii Bourd. & Galz.	—	—	—	1	—	—	—	1
Trechispora brinkmanii (Bres.) Rog. & Jacks.	—	1	—	—	—	—	—	1
Pholiota spectabilis Fr.	—	—	—	—	—	1	—	1
Stereum purpureum (Pers.) Fr.	—	—	—	1	—	—	—	1
Collybia velutipes Curt. ex Fr.	—	—	—	1	—	—	—	1
Polyporus tulipiferae (Schw.) Overh.	—	—	—	—	1	—	—	1
Hericium laciniatum Leers ex Banker	—	—	—	—	—	1	—	1
Total	7	22	42	64	60	65	12	272

It is evident that in stands younger than 140 years, butt rots accounted for a negligible volume of decay in the merchantable portions of the sampled trees. Although butt rots accounted for only 7.5% of the total rot volume, their importance is not fully indicated by these figures. If losses from wind breakage indirectly due to butt rot could have been tabulated, it is possible that total volume losses caused by butt rot would have equalled or perhaps even surpassed those caused by trunk rot.

Fungi Associated with Decay and Stain in Living Aspen

Christensen *et al.* have stated that *Fomes igniarius* is the principal cause of heart rot in aspen throughout the entire range of the tree (3). This was found to be the case in the Upper Pic region of Ontario, since *Fomes igniarius* var. *populinus* (Neu.) Campb. (hereafter referred to as *Fomes igniarius*) was isolated from nearly all of the samples collected of white spongy rot. Riley (8) has presented a detailed account of *Fomes igniarius* decay of aspen on the Petawawa Forest Experiment Station, Ontario.

The frequency with which different fungi were isolated from samples of yellow stringy trunk rot is presented in Table II. *Radulum casearium* (Morgan) Lloyd accounted for 226 or 83% of the 272 identified isolations obtained from these samples. *Corticium polygonium* Pers. was isolated 36 times from this type of rot; the majority of these isolations were from incipient decays (Figs. 2 and 3). Table II shows that in young aspen from 40 to 80 years of age, *C. polygonium* was isolated more frequently than *R. casearium*, though it was never isolated from trees older than age 120. *R. casearium* may therefore be designated as the probable cause of most of the characteristic advanced stringy trunk rot of aspen. The fruiting bodies of this fungus are yellow-brown, resupinate, with coarse teeth (Fig. 7). The decay-producing potentialities of the other fungi associated with this rot have not yet been determined. It is possible that the complex of fungi associated with yellow stringy trunk rot may explain the absence of any marked relationship between this type of decay and age (Table I).

Table III shows the frequency with which different fungi were isolated from stringy butt rots. Specific identifications were obtained from more than one third of the 530 butt rots encountered in this study, and the frequency of isolation provides a measure of the relative importance of the several fungi associated with this type of rot. It is apparent that *Pholiota spectabilis* Fr. and *Armillaria mellea* (Vahl ex Fr.) Quél., with 64 and 50 isolations respectively, accounted for 62% of the 185 isolations identified, and are probably the two principal causes of butt rot in aspen. The fruiting bodies of these two fungi are mushroomlike, and often develop in clusters on dead stumps or at the base of dying or dead trees. Of the remaining 13 organisms isolated from these rots, only three were isolated more than twice. These were *R. casearium* (29 isolations), *Pholiota adiposa* Fr. (19 isolations), and *Collybia velutipes* Curt. ex Fr. (10 isolations). The relative frequency with which these fungi were isolated was approximately the same for aspen in all age classes.

TABLE III

FUNGI ISOLATED FROM STRINGY BUTT ROT OF ASPEN AT DIFFERENT AGE CLASSES IN THE UPPER PIC REGION OF ONTARIO

Organism	Number of isolations							
	Age class							
	41–60	61–80	81–100	101–120	121–140	141–160	161–180	Total
Pholiota spectabilis Fr.	—	3	27	3	6	19	6	64
Armillaria mellea (Vahl ex Fr.) Quél.	1	2	20	2	4	20	1	50
Radulum casearium (Morgan) Lloyd	—	—	3	9	1	12	4	29
Pholiota adiposa Fr.	—	—	4	1	3	8	3	19
Collybia velutipes Curt. ex Fr.	—	—	3	—	2	4	1	10
Odontia bicolor (Alb. & Schw. ex Fr.) Bres.	—	—	—	—	—	1	1	2
Pleurotus ostreatus Jacq. ex Fr.	—	—	—	—	—	1	1	2
Polyporus versicolor L. ex Fr.	—	—	—	—	—	1	1	2
Daedalea unicolor (Bull.) Fr	—	—	—	—	—	1	—	1
Polyporus adustus Willd. ex Fr.	—	—	1	—	—	—	—	1
Coprinus micaceus Fr.	—	—	—	1	—	—	—	1
Polyporus tulipiferae (Schw.) Overh.	—	—	—	—	—	1	—	1
Fomes pinicola (Sw.) Cooke	—	—	1	—	—	—	—	1
Stereum murraii (Berk. & Curt.) Burt.	—	—	—	—	—	—	1	1
Trechispora raduloides (Karst.) Rogers	—	—	1	—	—	—	—	1
Total	1	5	60	16	16	68	19	185

The fungus most consistently isolated from wood with red mottled stain was a species of *Libertella*. Fritz (4) has shown that this member of the Fungi Imperfecti is capable of attacking sound, autoclaved poplar wood. Several other fungi were found associated less often with this stain. *Corticium polygonium* and another member of the Fungi Imperfecti, *Phialophora alba* van Beyma, were isolated from the red mottled stain which occurred throughout the trunks of young (30 to 50 years) aspen. In overmature trees, two fungi, *Polyporus adustus* Willd. ex Fr. and *Stereum purpureum* (Pers.) Fr., were isolated occasionally from red stained wood. Nevertheless, *Libertella* sp. accounted for over 60% of the identified isolations obtained from red mottled wood, and appeared to be the only fungus which was associated with this stain in aspen of all ages.

Corticium polygonium was consistently isolated from the light-brown stain frequently encountered as the only interior defect in young aspen. This is to be expected, since this stain apparently develops into the incipient yellow stringy decays from which *C. polygonium* was commonly isolated (Table II). *C. polygonium* and other organisms were isolated from the brown stain surrounding heart rots in older aspen, some of the others being *Phialophora alba*, *Libertella* sp., *F. igniarius*, and *R. casearium*. The two latter fungi were particularly abundant in the brown stains of aspen older than 100 years, indicating that these dark greyish-brown stains which often surround advanced trunk rots in older trees may be caused in part by radial extensions of these advanced decay organisms. Nevertheless, as in the case of *Libertella* sp. and red mottled stain, *C. polygonium* was the only fungus consistently isolated from brown stain in aspen of all ages. Although *C. polygonium* did not appear to be associated as commonly with brown stain in the old trees as in young trees, it accounted for over 50% of the identified isolations made from brown stain of aspen.

Entrance of Fungi and External Indications of Decay

It is sometimes difficult to ascertain the exact mode of entrance of a fungus believed to have caused heart rot in a living tree. For this reason definite figures for the different avenues of entrance associated with decay cannot be given. The results are based upon general observations, and upon the majority of decays which fortunately permitted the determination of the point of entrance of the associated fungi with reasonable accuracy.

The majority of fungi connected with butt-rot infections in aspen undoubtedly entered through the roots. It seems likely that the five fungi associated with most of the butt decay in aspen can and do exist throughout much of the root system. Since aspen occurs in relatively pure, dense stands with intermingling root systems, the breakage of small rootlets because of frost heaving and wind bending would provide good entrance points. There can be little doubt that many of the 1224 trees recorded as sound at stump height actually were infected by butt-rot fungi in the roots and in the bole beneath stump height. Stringy butt rots were associated with each of the 56

TABLE IV

THE RELATION BETWEEN THE NUMBER OF VISIBLE *Fomes igniarius* FRUITING BODIES AND THE OCCURRENCE OF *F. igniarius* DECAY IN ASPEN

Age class	Number of trees sampled	Number of trees with *F. igniarius* decay	Number of trees with one or more visible *F. igniarius* fruiting bodies	Percentage of trees with *F. igniarius* decay with fruiting bodies
41– 60	131	18	11	61.1
61– 80	271	52	41	78.8
81–100	553	183	150	82.0
101–120	357	179	151	84.4
121–140	165	100	86	86.0
141–160	238	170	166	97.6
161–180	39	29	25	86.2

TABLE V

THE RELATION BETWEEN AGE, THE INCIDENCE, AND THE VOLUME OF DECAY IN ASPEN IN THE UPPER PIC REGION OF ONTARIO

Age class	Average tree age	Number of trees sampled	Trees with decay		Total volume of trees sampled, cu. ft.	Total volume of rot within the merchantable portion of sampled trees, cu. ft.	Average volume of rot within the merchantable portions of infected trees, cu. ft.	Percentage of the total tree volume decayed within the merchantable portion of the sampled trees	
			Number	Percentage				Actual	Curved
41– 60	48	131	35	26.7	1,000.9	10.1	0.3	1.0	1.0
61– 80	71	271	130	47.8	3,989.3	108.6	0.9	2.7	2.8
81–100	92	553	321	58.0	9,422.2	466.9	1.5	5.0	4.9
101–120	111	357	313	88.9	7,501.9	606.9	1.9	8.1	7.3
121–140	128	165	149	90.3	5,488.1	438.1	2.9	8.0	10.1
141–160	150	238	225	94.5	10,300.1	1,504.6	6.7	14.6	13.5
161–180	166	39	39	100.0	1,644.1	276.2	7.1	16.8	17.3
Total		1,754	1,212		39,346.6	3,411.4			
Average				69.3			2.8	8.7	

basal scars encountered in the first 24 sample plots, indicating that a minority of butt-rot infections probably originated from such basal wounds as fire scars, frost cracks, and basal branch stubs.

The fungi connected with trunk rots of aspen also appear to be limited to one method of entrance. Approximately 90% of these rots could be traced to dead, broken branch stubs. As an aspen tree ages, the lower branches continually die and break off, providing direct passages for decay-causing fungi through the resistant sapwood into the heartwood (Figs. 1 and 2). A few trunk-rot infections originated in forked crowns, frost seams, and mechanical injuries. Seventy-five aspen with pronounced trunk wounds were encountered on the first 24 sample plots; of these, 63 or 84% had extensive heart rot. Thus, visible trunk wounds in aspen are fairly reliable indicators of heart rot in this species.

The most reliable external indication of decay in aspen is unquestionably the appearance of *Fomes igniarius* fruiting bodies (2). These fairly large, woody fructifications with black, wrinkled upper surfaces and brown, lower pore surfaces often project from dead branch stubs throughout the length of the bole. The occurrence of such a series of conks on a single tree, or of conks greater than four inches in width, was invariably associated with extensive *F. igniarius* heart rot. The close relationship between the number of visible *F. igniarius* fruiting bodies and the occurrence of *F. igniarius* decay in aspen stands older than 60 years is shown in Table IV.

The small, white, resupinate fruiting bodies of *Corticium polygonium* have been observed on or near dead branch stubs of aspen. These were difficult to detect, but when discovered they were usually found to be associated with extensive heart rot.

The Relation between Age and Decay

Since the number of infection courts and the time they have been effective increase with increasing age of the trees, it follows that the amount of heart rot will also increase with age. From Table V it is evident that this is so in aspen in the Upper Pic region of Ontario. The percentage of trees with heart rot in the merchantable portion of the bole increased steadily from 26.7% in stands at age class 41–60 to 100% in stands at age class 161–180. Many of the decayed trees in young stands had only small traces of decay, whereas the majority of infected trees in older stands had extensive heart rot. The average volume of heart rot encountered in decayed aspen in stands within the 41–60 age class was only 0.3 cu. ft., compared with approximately 7 cu. ft. per infected tree in stands older than 140 years. For this reason, not only the number of infections but also the volume of heart rot must be considered if a reasonable assessment of the relationship between age and decay is to be made. The pronounced increase in the percentage of the total volume of trees decayed within the merchantable portions of the boles with increasing stand age is evident from the last two columns of Table V.

TABLE VI

THE RELATION BETWEEN AGE, SITE, AND THE PERCENTAGE OF DECAY IN ASPEN IN THE UPPER PIC REGION OF ONTARIO

	Percentage of the total tree volume within the merchantable portions recorded as decay*				Percentage of the total tree volume within the merchantable portions recorded as butt rot			
Age class	Site 1	Site 2	Site 3	Site 4	Site 1	Site 2	Site 3	Site 4
41– 60	2.1 (57)		0.4 (74)		0.00		0.14	
61– 80	1.8 (127)	2.9 (68)		3.7 (76)	0.06	0.09		0.10
81–100	4.1 (75)	5.8 (138)	3.6 (245)	7.1 (95)	0.90	0.77	0.32	0.35
101–120	8.9 (80)	13.4 (24)	7.7 (136)	6.7 (117)	0.46	0.37	0.36	0.86
121–140			8.3 (106)	7.7 (59)			0.57	0.42
141–160	17.6 (57)	14.6 (51)	12.6 (108)	14.4 (22)	2.13	0.74	1.07	0.98
161–180	15.8 (22)	18.1 (17)			4.60	4.79		

* Numbers of trees sampled are shown in brackets.

The Relation between Site and Decay

The growth and yield of a forest stand is undoubtedly related to the nature of the soil. There is much conflicting evidence, however, concerning the effect of soil conditions upon the extent of heart rot in trees (11). This is not surprising when one considers that trunk rots, which generally account for a far greater proportion of the decay volume than butt rots, originate mainly at branch stubs or trunk wounds and hence are less likely to be directly influenced by soil characteristics. Nevertheless, indirect influences of the nature of the soil on the progress of trunk rots through changes in tree growth rate, vigor, moisture content, and stand density should not be overlooked.

In forestry, the capacity of an area to support forest growth is referred to as site. Although all environmental factors should be considered in determining the site of an area, it is generally agreed that the availability of soil moisture is of prime importance. In assessing the influence of this factor upon the extent of heart rot in aspen in the study region, detailed examinations of the soil conditions were made in soil pits dug to a depth of about four feet, or to bedrock, on each of the 47 sample plots.

Aspen stands occur only within a fairly definite range of soil conditions throughout northern Ontario. The soil types or sites on which aspen stands occurred in the study region were arbitrarily divided, with the assistance of foresters of the Marathon Paper Mills of Canada, Limited, into four categories, based on the site classification system designed by Hills for the forests of Ontario (6). Brief descriptions of the soils of each of these four sites follow:

Site 1: Moisture regime 1 (dry). Slightly silty or fine pervious sands, or coarse mixtures of sand and pebbles. Fast drainage.

Site 2: Moisture regime 2 (adequate). Permeability 1 or 2 (good). Medium, silty or loamy sands over pervious sands or coarse material.

Site 3: Moisture regime 2 (adequate). Permeability 3 to 5 over 9 (fair to poor). Deep sandy silts and loams, or shallow loams over bedrock.

Site 4: Moisture regime 4 (moist). Deep silty loams and clays, or sandy loams over somewhat impervious clay.

The average height of the dominant trees, average tree volume, and the total volume of the merchantable aspen per acre were computed for each sample plot. The analysis of these data indicated that the growth and yield of aspen were approximately the same on all four sites. The relationship between age of stand, site, and volume of decay is presented in part 1 of Table VI. Variations in the extent of decay on the four sites are not great, nevertheless Table VI shows that aspen stands located on deep, sandy silts or loams, or on shallow sandy loams over impervious clay (site 3), are generally less defective than stands located on drier (1 and 2) or wetter (4) sites, particularly the former. Stoeckler (9) arrived at somewhat similar conclusions regarding aspen stands in Wisconsin and Minnesota.

Part 2 of Table VI presents the results of an analysis of the relationship between site based on soil conditions and the volume of butt rot in the merchantable portions of aspen. This table shows a distinct tendency for

aspen to have a greater volume of butt rot on dry sites (site 1) than on any of the other sites. Further investigations on the effect of soil moisture and root moisture content upon the development of fungi that cause butt rot in aspen are desirable. The author has reported that the opposite relationship between site and butt rot exists in balsam fir (1), and similar results were reported by Heimburger and McCallum (5). Peace (7) in England found that butt rot was more abundant in conifers on moist, clay soils, while in larch the relationship was reversed. The fact that aspen and larch grow well on fairly moist soils, whereas balsam fir and pine prefer drier, well-drained soils, suggests that a possible explanation of these relationships may be that the susceptibility of the host to butt-rot infection is increased when grown on sites other than those which allow optimum growth and development.

Discussion

The mycological data presented in this paper suggest that the heartwood of living aspen is invaded and colonized by one or more fungi which are not generally associated with advanced decay prior to the development of advanced heart rot. *Corticium polygonium*, *Libertella* sp., and *Phialophora alba* were frequently isolated from heartwood stains and incipient decays in young aspen, from stained wood adjacent to advanced cores of heart rot in mature and overmature aspen, from branch stubs, and from heartwood stains above and below columns of advanced heart rot. Hence, these organisms are associated with stain and incipient decay in the regions of the trunk which are likely to be subsequently invaded by the organisms involved in advanced rot, primarily *Fomes igniarius* and *Radulum casearium*.

Further investigations are warranted into the exact nature of this apparent succession of fungi in the decay of living aspen heartwood. It is probably safe to assume that all of these fungi are capable of reaching the substrate at approximately the same time. There was no evidence of any marked competitive or synergistic interactions among the fungi. It would appear likely that the sequence of fungi in aspen heartwood results from changes in certain properties of the wood substrate. Hence, the properties of the heartwood of recently killed aspen may permit only the "preliminary fungi" to colonize the substrate, whereas the establishment of the "principal fungi" is delayed until certain properties of the substrate have been altered. Laboratory tests should indicate whether or not the "preliminary fungi" play significant roles in bringing about these changes.

The foregoing discussion of a possible metabiotic series in the decay of aspen heartwood suggests that members of the Fungi Imperfecti group may play more important roles in the decay process of living trees than has heretofore been recognized. In past cultural studies of tree decays, investigators have in many cases disregarded isolations of Fungi Imperfecti, considering them to be contaminants or to have little significance in the decay process. Survey data concerning fungi isolated from stains and decays of approximately 2500 aspen sampled throughout the province of Ontario have recently been

assembled. These indicate that the two Fungi Imperfecti, *Libertella* sp. and *P. alba*, isolated so consistently from aspen in the Upper Pic region, occurred with approximately the same frequency and were associated with the same stains in aspen throughout Ontario. Many other fungi of this group were consistently, though less frequently, isolated from these trees. Laboratory inoculation tests, which may clarify to some extent the roles of these fungi in the decay process of aspen, will be carried out.

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STUDIES ON THE FUSARIA WHICH CAUSE WILT IN MELONS

II. THE EFFECT OF LIGHT, NUTRITION, AND VARIOUS CHEMICALS ON THE SPORULATION OF CERTAIN FUSARIAL ISOLATES, AND PRELIMINARY INVESTIGATIONS ON THE ETIOLOGY OF WILTING OF THE MUSKMELON FUSARIUM[1]

JAMES REID

Abstract

Light was shown to affect the amount of macrospore production in a number of *Fusarium* species. Macrospore production and the ratio of macrospores to microspores increased with increasing light intensity and vice versa. Only colony areas exposed to light during active growth produced macrospores on potato dextrose or Czapek's agars. Light also greatly stimulated macrospore production on V-8 vegetable juice agar. Both carbon and nitrogen sources were also important in determining the kind and amount of sporulation in the species tested. Colony growth was appressed in light, and pigmentation of the mycelium often was produced in response to light.

Various chemicals, known to be enzyme inhibitors, induced different effects on growth and sporulation, depending on the substrate used as the carbon source. For example, sodium fluoride inhibited growth and sporulation when glucose was the substrate, but was without effect when sodium acetate, which supports equally vigorous growth, was the substrate.

Studies on the etiology of wilting indicated that the muskmelon *Fusarium* produces at least three chemical fractions which may contribute to wilting. One was thermostable, non-dialyzable, apparently a protein, and possibly an enzyme. The second was thermostable and was probably a low molecular weight carbohydrate. These two induced wilting in plant cuttings rather than in intact plants, whereas the third fraction induced wilting in plants with undamaged root systems, and was thermostable, partially dialyzable, and stable through a wide pH range. Appropriate tests, however, indicated also that translocation through the vessels was significantly interfered with in plants showing incipient or more advanced wilting symptoms.

Introduction

The importance of sporulation in the fungi, and the reason for studying the factors influencing sporulation, have long been recognized (11).

Among the factors known to influence sporulation are the composition of the media, concentration of the media, temperature, light, mitotic poisons, chelating agents, and other factors. This paper is, in part, a study of the effect of light, nutrition, and certain chemical compounds on the sporulation of certain fusarium isolates.

The etiology of wilting has been under investigation recently in relation to fusarial diseases of other plants. Toxic substances have been demonstrated, but the nature of these and their action have been and are still disputed. In this paper are reported the results of preliminary investigations re the production of a wilting principle by the muskmelon *Fusarium*, *F. oxysporum* f. *melonis* (Leach and Curr.) Snyder and Hansen.

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Contribution from the Department of Botany, University of Toronto, Toronto, Ontario. Based on a thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy.

The Effect of Light on Sporulation and Growth of Various *Fusarium* Isolates

Considerable literature has accumulated dealing with the influence of radiation on the fungi, and this has been extensively reviewed by Harter (9) and Coons (2). Apparently, only rarely in the genus *Fusarium* has much attention been given to the direct influence of light and dark on the size of spores, or to the increase in number of specific types of spores in response to known periods or intensities of light experience.

The following experiments were designed to investigate the effect of light on sporulation in terms of the presence or absence of macrospores and microspores and the persistence of light effects on cultures of various ages.

All isolates used in these investigations were of monosporous origin and were maintained in soil tubes as the best way to insure genetic stability in the stock cultures. The three culture media used were Czapek's, potato dextrose, and V-8 vegetable juice agars.

All experiments were carried out in chambers in which temperature and humidity could be held at any desired level. The light source consisted of varying numbers of General Electric 40-w. standard warm white fluorescent light bulbs, depending upon the desired level of illumination. Illumination was measured by a Weston illumination meter, model 756, and the temperature and moisture recorded throughout the experiments by means of a Bendix hydro- thermograph. Dark controls for the illuminated cultures consisted of at least two cultures of any fungus under test, maintained in copper cans which were fitted inside with metal supports in such a way that the lower half of the cans remained empty.

The results are reported on the basis of the number of the different types of spores per square centimeter of colony growth. A determination of spores was carried out: the test plates were flooded with a known amount of water containing a few milliliters of aerosol as a wetting agent and the colony rubbed with an inoculating loop of heavy weight. The liquid was then filtered through a single layer of cheesecloth and the filtrate collected. Each plate was treated in this manner three times to insure removal of as many spores as possible. The combined filtrates were then adjusted to the original volume of water used and the number of spores determined for each plate of a series by means of a Levy haemocytometer counting chamber. Four counts were made for each plate and the counts for all plates of an isolate in any series were then averaged.

The following organisms were used in this study: (1) *Fusarium udum* Butler, (2) *Fusarium solani* (Mart) App. & Wr., (3) *Fusarium oxysporum* f. *niveum* (E.F.S.) Snyder and Hansen, (watermelon), (4) *Fusarium oxysporum* f. *melonis* Leach & Curr. Snyder and Hansen, (muskmelon), (5) *Fusarium oxysporum* f. *conglutinans* (Wr.) Snyder and Hansen, (cabbage), (6) *Fusarium oxysporum* f. *callistephi* (Beach) Snyder and Hansen, (aster), (7) *Fusarium oxysporum* f. *tracheiphilum* (E.F.S.) Snyder and Hansen, (cowpea), (8)

Fusarium sp. from pea plants, (9) and (10) two *Fusarium* species from navy bean plants.[2] Nos. 1, 2, 6, 8, 9, and 10 were donated by Dr. J. J. Miller.

No. 8 is a *Fusarium* originally isolated from pea, which has not been named, since on the basis of host relationship and symptomatology it appears to belong to the section Martiella. On the basis of morphology, however, it closely resembles fusaria of the section Elegans. In the following tables most of the various fusaria are listed in terms of their host plant.

The studies included the effect on the kind and amount of sporulation of cultures (*a*) grown in continuous light and continuous darkness, temperature and humidity constant; (*b*) grown under alternating periods of light and darkness; (*c*) illuminated after various growth experiences; (*d*) grown under various light intensities; and (*e*) subjected to temperature changes at constant light intensity.

(*a*) *The Effect on the Kind and Amount of Sporulation of Cultures Grown in Continuous Light or Continuous Darkness for 144 Hours, with Temperature and Humidity Constant*

In these experiments all three media were employed. The light source consisted of eight fluorescent light bulbs, giving a light source of 1600 ft-c. The temperature varied between 72 and 74° F., and the relative humidity, between 45 and 50%.

From the results of Table I it is apparent that *F. solani* growing on Czapek's agar produced macrospores in both light and darkness, but the number of macrospores per square centimeter was greatly increased on exposure to light. The other nine organisms produced no macrospores when grown in the dark on Czapek's agar, whereas macrospores developed in all cases when these cultures were exposed to light. The number of macrospores produced by each of the test organisms was also increased as a result of exposure to light when they were grown on both potato dextrose and V-8 vegetable juice agars. In the absence of light, where potato dextrose agar was employed, none of the dark control cultures produced macrospores, whereas, when V-8 vegetable juice agar was used, six of the isolates produced macrospores in the dark control cultures. Figures 1–16 illustrate the spores produced by *F. solani*, *F. udum*, the muskmelon *Fusarium*, and the pea *Fusarium*, when grown on potato dextrose agar in light and in darkness, and when grown on V-8 vegetable juice agar and Czapek's agar in light.

On all three media employed, it is evident that the organisms tested either began producing macrospores or greatly increased their rates of macrospore production on exposure to light. Further, it is clear that at least in some fusaria nutrition is an important factor conditioning production of macrospores when light is absent. It is apparent that a medium such as V-8 vegetable juice agar is able to induce some degree of macrospore production in darkness, although the numbers of macrospores produced do not approach those produced following light stimulation.

[2]The numbers (4) and (5) applied to the navy bean isolates in the text from here on are the isolate designations of the donor, Dr. J. J. Miller.

TABLE I

A COMPARISON OF MACROSPORE AND MICROSPORE PRODUCTION BY SINGLE SPORE COLONIES OF FUSARIUM ISOLATES IN LIGHT AND DARKNESS ON THREE MEDIA

Organism	Light (1600 ft-c.) or dark experience	Czapek's agar			Potato dextrose agar			V-8 vegetable juice agar		
		Macrospores*	Microspores*	Ratio macrospores to microspores	Macrospores	Microspores	Ratio macrospores to microspores	Macrospores	Microspores	Ratio macrospores to microspores
Pea	L	1.8	9.3	0.19	1.34	2.4	0.56	5.55	1.2	4.62
Fusarium	D	0	5.2	0	0	7.95	0	0.08	12.3	0.07
Cabbage	L	2.3	3.5	0.66	0.67	2.92	0.23	1.88	4.4	0.44
Fusarium	D	0	2.6	0	0	0.38	0	0.03	6.1	0.005
Navy bean	L	3.58	2.3	1.55	0.55	0.5	1.1	3.62	1.1	3.28
Fusarium (4)	D	0	7.8	0	0	0.32	0	1.16	14.6	0.08
Watermelon	L	0.79	0.71	1.12	0.65	0.98	0.66	4.2	0.97	4.3
Fusarium	D	0	0.07	0	0	0.62	0	0.02	4.17	0.005
Aster	L	0.06	0.88	0.07	19.6	11.2	1.75	3.28	0.76	4.3
Fusarium	D	0	0.79	0	0	0.97	0	0.04	5.15	0.008
Navy bean	L	0.13	0.74	0.18	1.38	3.19	0.43	1.54	0.3	5.1
Fusarium (5)	D	0	0.2	0	0	3.19	0	0	2.13	0
Cowpea	L	0.66	1.68	0.39	1.17	15.9	0.07	3.62	0.85	4.25
Fusarium	D	0	1.77	0	0	1.77	0	0	6.96	0
Muskmelon	L	1.38	1.22	1.14	1.08	1.2	0.9	6.3	1.15	5.5
Fusarium	D	0	0.21	0	0	0.89	0	0	4.0	0
Fusarium udum	L	0.25	0.61	0.42	0.87	0.62	1.4	2.58	0.43	6
	D	0	0.03	0	0	0.91	0	0	1.29	0
Fusarium solani	L	2.73	0.53	5.2	2.28	0.53	4.3	3.89	0.49	7.9
	D	0.25	4.2	0.06	0	3.12	0	1.05	9.6	0.11

*To get the total number of spores per square centimeter, multiply the numbers in these columns by 10^6.

On Czapek's and potato dextrose agars there was no consistent correlation between the number of microspores and macrospores produced in the light and in the dark. In some isolates the number of microspores increased in the dark; in other cases the number decreased. On the V-8 vegetable juice agar, as the number of macrospores decreased to zero in the dark, the number of microspores increased.

(*b*) *The Effect on Growth and Sporulation of Alternating Periods of Light and Darkness*

The medium employed in this experiment was Czapek's agar and the experimental procedure was similar to that described in section (*a*).

One set of cultures for light exposure, and one set for dark control, were placed in each chamber; one chamber was darkened at the start of the experiment while the other was fully illuminated by light of 1600 ft-c. light intensity. Automatic time switches turned the lights on and off at 12-hour intervals. The light experience in chamber 1 consisted of alternating periods of light and darkness, the first period being one of light. The second chamber was also illuminated and darkened alternately for 12-hour periods, but the first 12-hour period was one of darkness.

The colonies exposed to alternating light and dark intervals were definitely zoned. Concentric rings were apparent; the rings corresponding to light illumination were usually flesh ocher in color (31), with more compact mycelia than the rings corresponding to the darkened periods, which were made up of mostly white to occasionally cartridge buff aerial mycelium.

From the results of this experiment, it was apparent that, with the exception of *Fusarium solani*, only cultures exposed to light produced macrospores, and that *Fusarium solani* produced a greater number of macrospores in the light than in the dark.

With 7 of the 10 organisms tested, the number of microspores produced on the cultures which started their growth in the light was greater than on the cultures which started their growth in the dark. Two other organisms, the muskmelon *Fusarium* and *Fusarium udum*, produced nearly equal numbers of microspores with both light experiences.

In every case, more macrospores were produced by the cultures started in the dark than by the corresponding cultures started in the light. More striking than the change in actual numbers, however, was the change in the ratio of macrospores to microspores per square centimeter, which was higher in every case in the cultures started in the dark. Apparently, although the cultures had equal total exposures to light and darkness, during equal periods of time those started in darkness must have had a greater culture area exposed to light than those cultures started in the light.

(*c*) *The Effect of Light on the Sporulation of Cultures Illuminated after Various Growth Experiences*

Harter (9) reported that fusarium cultures exposed to light for 4 days and then placed in darkness retained their ability to produce macrospores through-

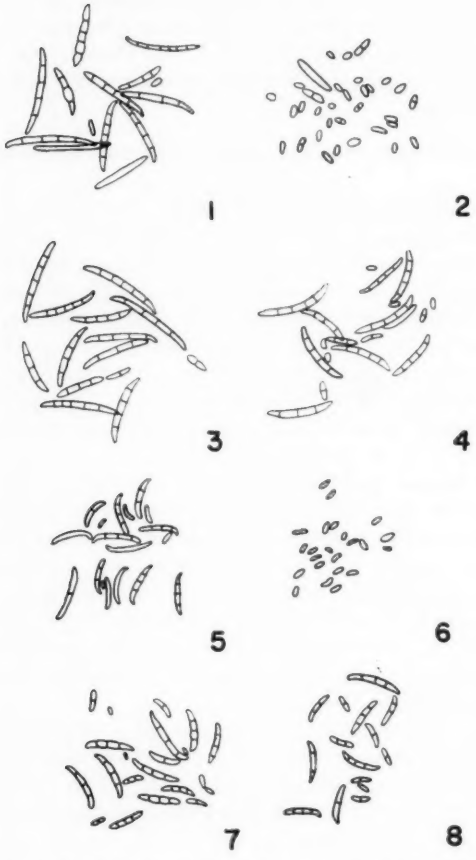

FIG. 1. Spores produced by *F. solani* growing on potato dextrose agar in response to light. Camera lucida drawings ×202.
FIG. 2. Spores produced by *F. solani* growing on potato dextrose agar in darkness. Camera lucida drawings ×202.
FIG. 3. Spores produced by *F. solani* growing on V-8 vegetable juice agar in response to light. Camera lucida drawings ×202.
FIG. 4. Spores produced by *F. solani* growing on Czapek's agar in response to light. Camera lucida drawings ×202.
FIG. 5. Spores produced by *F. udum* growing on potato dextrose agar in response to light. Camera lucida drawings ×202.
FIG. 6. Spores produced by *F. udum* growing on potato dextrose agar in darkness. Camera lucida drawings ×202.
FIG. 7. Spores produced by *F. udum* growing on V-8 vegetable juice agar in response to light. Camera lucida drawings ×202.
FIG. 8. Spores produced by *F. udum* growing on Czapek's agar in response to light. Camera lucida drawings ×202.

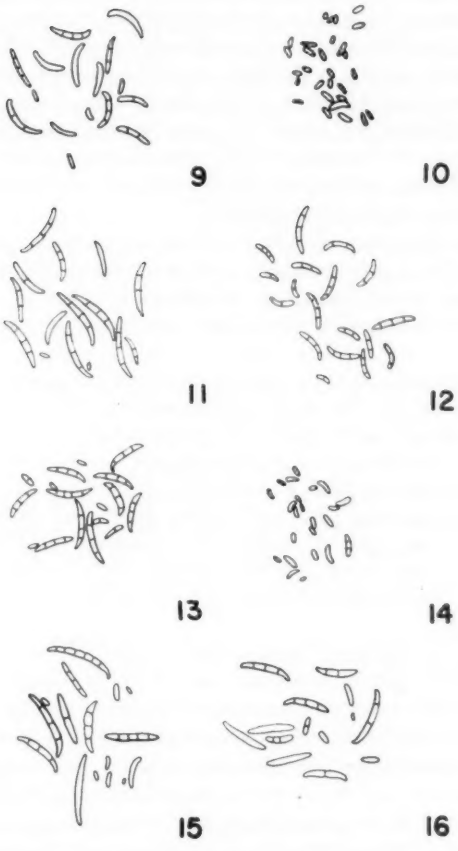

FIG. 9. Spores produced by muskmelon *Fusarium* growing on potato dextrose agar in response to light. Camera lucida drawings ×202.

FIG. 10. Spores produced by muskmelon *Fusarium* growing on potato dextrose agar in darkness. Camera lucida drawings ×202.

FIG. 11. Spores produced by muskmelon *Fusarium* growing on V-8 vegetable juice agar in response to light. Camera lucida drawings ×202.

FIG. 12. Spores produced by muskmelon *Fusarium* growing on Czapek's agar in response to light. Camera lucida drawings ×202.

FIG. 13. Spores produced by pea *Fusarium* growing on potato dextrose agar in response to light. Camera lucida drawings ×202.

FIG. 14. Spores produced by pea *Fusarium* growing on potato dextrose agar in darkness. Camera lucida drawings ×202.

FIG. 15. Spores produced by pea *Fusarium* growing on V-8 vegetable juice agar in response to light. Camera lucida drawings ×202.

FIG. 16. Spores produced by pea *Fusarium* growing on Czapek's agar in response to light. Camera lucida drawings ×202.

out the duration of their growing period. Normal spore production (32) occurred principally in those zones of growth which were initiated in light, or in which development took place during continued exposure to light.

The purpose of this experiment was to investigate whether fusarium cultures which started their growth in the light would retain their ability to produce macrospores after being returned to the dark. Also, young cultures grown in the dark for different lengths of time were exposed to light to see whether mycelium produced in the dark would produce macrospores on exposure to light, or if macrospore production was limited only to mycelium which had light experience during its development.

Four series of plates poured with Czapek's agar were inoculated. Two of the series, A and B, were inoculated with germinating single spores, one per plate. A second set of plates was inoculated by transfer of small bits of mycelium from a 2-day-old stock culture, previously prepared from the soil tube stocks.

The experiment was actually carried out in four separate sections owing to the limited size of the constant-temperature chambers. In the first section, pea, cabbage, navy bean (4), watermelon, and aster fusaria were inoculated into two sets of 70 plates each, 14 for each isolate by the two previously mentioned inoculation methods. All the plates were then placed in the two constant-temperature chambers which had previously been set at the same temperature, humidity, and intensity of illumination. The plates with the single spore type of inoculum were placed in one chamber, while the plates inoculated with bits of mycelium were placed in the second chamber.

At intervals of 24, 48, and 72 hours three plates of each isolate were removed from each chamber, placed in copper cans of the previously described type, and returned to the constant-temperature chambers for the duration of the experiment. The total time for the experiment was 144 hours.

In the second section the navy bean (5), cowpea, and muskmelon *Fusaria*, and *Fusarium solani* and *Fusarium udum* were treated in the same manner.

In sections 3 and 4, the grouping of the isolates into two lots of five was retained, but here all the plates were in copper cans at the start of the experiment. After intervals of 24, 48, and 72 hours, three plates of each isolate were removed from the cans and exposed to light. Inoculation was by the two previously mentioned methods.

In the experiment, there were then: (1) single spore cultures started in the light, and placed in darkness at various intervals, (2) cultures started from mycelial inoculation and treated as in (1), (3) single spore cultures started in darkness and placed in light after various intervals, (4) cultures started from mycelial inoculation and treated as in (3), (5) single spore and mycelial-inoculated cultures grown entirely in the light, and (6) single spore and mycelial-inoculated cultures grown entirely in the darkness.

The cultures which remained in the light throughout the experiment were appressed, with some of the isolates taking on a very slimy appearance, owing

to the abundance of the macroconidia produced. The cultures which remained in the dark for the entire time exhibited good mycelial growth, ranging from white to cartridge buff (31), depending upon the isolate, but no macrospores were found. In all cases the results with single spore inoculations supported the thesis that only in those areas of the colonies which were exposed to light during their period of development were macrospores produced in abundance. Contrary to the findings of Snyder and Hansen (32), microspores were again produced abundantly in the dark.

On the plates inoculated with single spores and treated in both manners, a graded response in sporulation was found depending upon the length of exposure to light. The longer the plates were allowed to remain in the light, the greater was the area which produced both macrospores and microspores; and the longer the plates were allowed to remain in the dark before being placed in the light, the greater was the area producing only microspores. No residual carry-over effect of light was observed.

A residual effect was found in some of the cultures originally grown in the dark. Cultures which had been inoculated by mycelial transfer and placed in the light after growing for 24 or 48 hours in darkness did show some macrospore production in the areas which had grown in darkness. When the dark period was 72 hours before the transfer to light was made, however, macrospores were not produced in the areas which had grown originally in darkness.

After the 24- and 48-hour dark intervals, it is possible that enough new growth occurred in these areas during exposure to the light to account for the macrospores produced. This would not explain why the cultures started from single spore inoculations, with the same light experience, produced macrospores only in those areas of the colony which developed in the light.

(*d*) *The Effect of Various Light Intensities on Sporulation of 10 Fusarial Isolates*

The purpose of this experiment was to investigate whether an increase or a decrease in light intensity would have a corresponding effect on the numbers of the two types of spores produced.

Since cultures growing on V-8 vegetable juice medium were apparently more sensitive to light, as witness the greater macrospore yield on this medium in response to light, this medium was employed. Plates were poured with V-8 vegetable juice medium, inoculated at the center of the plate by the single spore method, and placed in constant-temperature chambers. The temperature varied between 72 and 74° F., and the relative humidity, between 46 and 51% throughout this experiment The intensity of the light source was varied by changing the number of light bulbs, or adjusting the distance of the cultures from the light source. For the lowest light intensity a 15-w. General Electric warm white fluorescent bulb was used.

The light intensities tested were 1600, 1200, 800, 500, 240, 80, 40, and 20 ft-c.

In Table II the results with the 1600, 240, 80, 40, and 20 ft-c. intensities are reported. The results with the 1200, 800, and 500 ft-c. intensities were omitted because they were the same as those with the 1600 ft-c. intensity.

TABLE II

A COMPARISON OF MACROSPORE (M) AND MICROSPORE (m) PRODUCTION BY SINGLE SPORE COLONIES OF 10 FUSARIUM ISOLATES ON V-8 VEGETABLE JUICE AGAR IN DARKNESS AND IN FIVE LIGHT INTENSITIES AFTER 144 HOURS

	1600 ft-c.			240 ft-c.			80 ft-c.			40 ft-c.			20 ft-c.			Dark control		
	Spores/sq. cm.		Ratio,	Spores/sq. cm.		Ratio,	Spores/sq. cm.		Ratio,	Spores/sq. cm.		Ratio,	Spores/sq. cm.		Ratio,	Spores/sq. cm.		Ratio,
Organism	M*	m*	M/m	M	m	M/m	M	m	M/m	M	m	M/m	M	m	M/m	M	m	M/m
Pea *Fusarium*	5.1	1.52	3.36	3.11	0.98	3.17	0.81	1.18	0.69	0.1	1.09	0.09	0.05	1.39	0.04	0.08	14.1	0.01
Cabbage *Fusarium*	1.87	4.9	0.38	0.72	4.47	0.16	0.73	4.69	0.16	0.42	4.05	0.1	0.03	0.61	0.05	0.03	6.39	0.01
Navy bean *Fusarium* (4)	3.42	1.21	2.83	5.06	2.6	1.95	3.06	2.07	1.48	1.71	7.61	0.22	0.75	8.1	0.09	1.21	13.6	0.09
Watermelon *Fusarium*	4.7	1.01	4.65	0.66	0.85	0.78	0.32	3.46	0.09	0.03	4.51	0.007	0.02	5.51	0.004	0.02	4.27	0.01
Aster *Fusarium*	3.39	0.78	4.35	0.82	2.47	0.33	0.05	0.17	0.29	0.05	1.01	0.05	0.04	3.14	0.01	0.04	5	0.01
Navy bean *Fusarium* (5)	1.73	0.31	5.58	0.4	0.55	0.73	0.11	3.9	0.03	0.002	1.02	0.002	0	1.41	0	0.02	2.18	0.01
Cowpea *Fusarium*	3.42	0.91	3.76	2.43	2.12	1.15	0.22	1.42	0.15	0.005	4.2	0.001	0.02	5.35	0.004	0	6.75	0
Muskmelon *Fusarium*	7.1	1.31	5.42	4.1	1.16	3.53	3.87	6.15	0.63	0.03	1.85	0.027	0	2.1	0	0	4.2	0
Fusarium udum	3.69	4.41	0.84	0.57	0.64	0.89	0.14	0.74	0.19	0.02	0.91	0.02	0	1.03	0	0	1.33	0
Fusarium solani	3.69	0.55	6.71	2.13	0.33	6.45	1.49	0.18	8.28	1.21	8.4	0.14	1.65	10.7	0.15	0.16	1.05	0.15

*To get the total number of spores per square centimeter, multiply the numbers in these columns by 10^6.

TABLE III

THE INFLUENCE OF TEMPERATURE ON IRRADIATED AND DARKENED FUSARIUM CULTURES

Organism	Number of macrospores per square centimeter of colony, millions*									
	Irradiated cultures, ° C.					Darkened cultures, ° C.				
	30	27.5	25	22.5	20	30	27.5	25	22.5	20
Pea *Fusarium*	1.65	1.31	0.91	0.71	0.54	0.13	0.09	0.08	0.07	0.07
Cabbage *Fusarium*	1.43	1.01	0.84	0.64	0.41	0.05	0.04	0.04	0.03	0
Navy bean *Fusarium* (4)	3.63	3.21	2.56	2.51	2.06	1.54	1.3	1.21	0.81	0.72
Watermelon *Fusarium*	1.01	0.71	0.36	0.23	0.1	0.04	0.04	0.03	0.03	0.01
Aster *Fusarium*	0.1	0.06	0.05	0.03	0.02	0.04	0.04	0.04	0.02	0
Navy bean *Fusarium* (5)	0.38	0.31	0.22	0.15	0.1	0.06	0.05	0.03	0	0
Cowpea *Fusarium*	0.32	0.28	0.26	0.25	0.21	0.03	0.02	0	0	0
Muskmelon *Fusarium*	4.64	3.67	3.47	3.01	2.96	0.01	0	0	0	0
Fusarium udum	0.33	0.28	0.16	0.14	0.09	0.02	0	0	0	0
Fusarium solani	3.89	3	2.01	2.31	1.49	0.4	0.31	0.17	0	0

*To get the total number of spores per square centimeter, multiply the numbers in these columns by 10^6.

The results with the 1600 ft-c. light intensity of this experiment were essentially similar to those at the same intensity in the first experiment of this section. This suggests a consistency of the light response of these isolates to a given light intensity.

When one considers the results of the experiments it is apparent that as light intensity decreased there was a corresponding decrease in the number of macrospores produced per square centimeter of colony area. Many of the isolates grown in the lowest light intensity did not produce any more macrospores than the dark control cultures. This clearly indicates that, under the conditions of our experiments, the number of macrospores produced depends upon light intensity, and at least with some of the isolates tested this has to be above the minimum value tested to support any such sporulation.

To investigate whether the type of medium employed would influence the sensitivity of the isolates to varying light intensities, a second series of cultures was prepared as before, except that Czapek's agar was employed. From the results it was apparent that when the organisms were grown on this medium they were less sensitive to light, since, even at the 240 ft-c. light intensity, many of the fusaria which produced abundant macrospores at that level of illumination on the V-8 medium failed to produce macrospores on the Czapek's medium, and those isolates which did produce macrospores gave much lower yields than on the V-8 medium. This is as one would expect from the results of the first experiment.

(*e*) *The Influence of Temperature on Irradiated and Darkened Fusarium Cultures*

Cultures were prepared by means of the single spore method. Seven plates were prepared for each isolate, four to be exposed to light and three to be held at the same temperature and humidity as the first four, except they were placed in copper cans as dark control cultures. The light intensity supplied was of 80 ft-c. intensity, the medium employed was V-8 vegetable juice agar, and the temperatures selected were 30, 27.5, 25, 22.5, and 20° C.

From the results in Table III it is evident that a 10° C. increase or decrease in temperature increased or decreased the number of macrospores produced in both the irradiated and dark control cultures. While the increase or decrease in numbers was slight in the dark control cultures, it was more pronounced in the irradiated cultures. In the irradiated cultures at a given light intensity increase in temperature did not increase the macrospore yield of all the isolates by the same ratio. From this it would appear that it is necessary to control the temperature at which cultures are growing during irradiation experiments, in order to separate the effects of temperature and radiation.

The Effect of Nutrition on Sporulation

The nature and concentration of various nutrients has long been recognized as an important factor in the sporulation of fungi. With many fungi the minimum concentration of nutrients which will permit mycelial growth is seldom sufficient for the production of spores (11), while the concentration

most favorable for vegetative growth may be too high for maximum spore production. Klebs (17) found exhaustion of nutrients favored spore production. Lilly and Barnett (19) feel that the concentration of nutrients in a medium may have a profound influence upon fruiting, and that the different types of fruiting (sexual and asexual) may have different requirements.

Many natural media are more favorable to sporulation of many fungi than are standard synthetic media. One reason for this is that synthetic media often contain glucose as the carbohydrate source. While this sugar is often effective in supporting good mycelial growth, nevertheless it often fails to induce sporulation. More complex polysaccharides, such as are found in natural media, often support relatively greater sporulation than vegetative growth.

Similarly all of the more simple carbohydrates are not equal in their ability to support active sporulation. For example, Hawker (11) found the number of perithecia produced by *Melanospora destruens* was influenced by the kind of sugar used.

Less attention has been paid to the effect of nitrogen compounds on fruiting. Some fungi, however, apparently need complex nitrogen compounds for fruiting, while others can fruit when simple nitrogen compounds are present.

(A) The Effect of Certain Concentrations of Glucose on Sporulation by Eight Fusarial Isolates

The organisms employed were pea, cabbage, navy bean (4), watermelon, aster, navy bean (5), cowpea, and muskmelon *Fusaria*. Yeast nitrogen base (Difco) plus various concentrations of glucose plus 2% agar were dispensed in Petri dishes, and the medium inoculated by loopfuls of spore suspensions of standardized strengths. Yeast nitrogen base is a chemically defined carbohydrate-free medium, recommended for use in yeast taxonomy, containing salts, trace elements, vitamins, amino acids, and 0.5% ammonium sulphate as the nitrogen source. It had been found to be a good nutrient medium for the growth of fusarium isolates in previous experiments. The glucose concentrations employed were 7.5, 5, 2.5, 1, 0.5, 0.1, and 0.05%. Cultures were incubated at 25° C. in diffuse light of 1600 ft-c. intensity.

From the results it was apparent that the maximum sporulation of seven of the isolates occurred at or near the 1% glucose concentration, while with the aster *Fusarium* maximum sporulation occurred at or near the 0.5% glucose. Above and below these points there was a decrease in the number of spores produced, with six of the eight isolates producing no spores at the 7.5% concentration. As the concentration was decreased from the optimum for spore production, macrospores were produced in increasing numbers, becoming more abundant at the lowest concentration with six of the eight isolates tested.

(B) The Effect of Certain Carbohydrate Compounds on Sporulation by Eight Fusarial Isolates

The isolates and the methods of inoculation and incubation employed were those used in the preceding experiment. Yeast nitrogen base was the medium employed with the compound to be tested added at a 1% concentration.

TABLE IV

THE EFFECT OF VARIOUS CARBON COMPOUNDS ON THE SPORULATION OF VARIOUS FUSARIUM ISOLATES

	Number of spores per square centimeter of colony, millions*										
	Pentoses		Hexoses				Disaccharides				
Organism	Arabinose	Xylose	Glucose	Galactose	Mannose	Fructose	Maltose	Cellobiose	Lactose	Melibiose	Sucrose
Pea	7.28	2.91	8.29	7.3	9.16	6.73	7.3	5.61	2.59	6.32	7.97
Fusarium	m	(M) m	m	(M) m	(M) m	m	m	m	(M) m	m	M m
Cabbage	5.5	4.56	9.8	5.25	12.1	5.87	5.67	4.6	1.2	7.65	7.08
Fusarium	m	(M) m	m	(M) m	(M) m	(M) m	m	m	(M) m	m	M m
Navy bean	6.21	3.64	6.21	6.96	7.48	7.13	0.84	7.48	1.44	6.93	8.86
Fusarium (4)	m	(M) m	m	(M) m	(M) m	M m	m	m	(M) m	m	m
Watermelon	0.36	0.05	0.401	0.12	0.53	0.24	0.33	0.22	0.204	0.52	0.217
Fusarium	m	M m	(M) m	M m	(M) m	(M) m	m	M m	(M) m	(M) m	(M) m
Aster	1.61	0.19	0.51	0.99	1.18	0.73	0.41	0.78	0.204	0.61	0.79
Fusarium	m	m	(M) m	m	m	m	m	m	(M) m	m	(M) m
Navy bean	0.76	0.27	0.88	0.73	1.42	0.92	0.77	0.41	0.34	0.85	0.7
Fusarium (5)	m	m	m	m	m	m	m	m	m	m	m
Cowpea	1.91	1.08	2.17	1.3	3.15	1.3	1.02	0.25	1.51	1.04	1.12
Fusarium	m	m	m	m	m	(M) m	m	m	m	m	(M) m
Muskmelon	0.41	0.21	0.72	0.27	1.01	0.35	0.66	0.39	0.36	0.64	0.3
Fusarium	(m)	m	(M) m	M m	(M) (m)	(M) m	M m	M m	(M) m	m	(M) m

	Trisaccharides	Polysaccharides			Others		
	Raffinose	Starch	Inulin	Dextrin	Sodium acetate	Glycine	Mannitol
Pea	7.36	5.07	8.54	5.9	0.65	5.61	8.03
Fusarium	m	(M) m	M m	(M) m	(M) m	m	m
Cabbage	4.6	2.3	10.6	2.59	0.88	4.6	4.72
Fusarium	m	(M) m	(M) m	(M) m	(M) m	m	m
Navy bean	7.48	3.35	7.26	8.4	2.01	7.48	7.91
Fusarium (4)	m	M m	M m	M m	m	M m	m
Watermelon	0.22	0.22	1.1	0.37	0.09	0.22	0.38
Fusarium	M m	M m	M m	M m	M m	M m	m
Aster	0.78	0.54	2.4	0.39	0.21	0.78	0.58
Fusarium	m	m	m	(M) m	m	m	m
Navy bean	0.41	0.81	2.03	0.32	1.57	0.41	0.59
Fusarium (5)	m	m	m	(M) m	(M) m	M m	m
Cowpea	2.01	1.28	2.24	1.24	2.12	0.47	1.05
Fusarium	m	(M) m	M m	(M) m	(M) m	m	m
Muskmelon	0.39	0.4	3.29	0.55	0.14	0.39	0.29
Fusarium	M m	(M) m	M m	(M) m	(M) m	m	m

*To get the total number of spores per square centimeter, multiply the numbers in these columns by 10^6.

NOTE: m = Microspores (m) = Sparse microspores Blank, no microspores or macrospores
M = Macrospores (M) = Sparse macrospores

M — Macrospores (M) — Sparse macrospores

TABLE V

THE EFFECT OF VARIOUS NITROGEN COMPOUNDS ON THE SPORULATION OF DIFFERENT FUSARIAL ISOLATES

Organism	Number of spores per square centimeter of colony, millions*						
	NH_4Cl	Asparagine	$NaNO_3$	Glycine	Ammonium tartarate	Urea	KNO_3
Pea	20.62	23.1	7.42	22.7	17.93	10.94	1.64
Fusarium	m	(M) m	m	M m	m	m	m
Cabbage	26.98	14.2	9.4	15.3	25.73	10.1	3.07
Fusarium	m	(M) m	m	M m	m	m	m
Navy bean	7.24	9.67	6.62	15.3	22.41	6.81	2.3
Fusarium (4)	m	(M) m	m	M m	M m	m	(M) m
Watermelon	0.19	0.26	0.49	0.14	0.16	0.06	0.17
Fusarium	m	m	(M) m	(M) m	m	M m	m
Navy bean	0.08	0.48	0.46	0.67	0.53	0.85	0.89
Fusarium (5)	m	m	m	m	m	m	m
Cowpea	0.12	2.52	1.15	2.4	1.5	0.36	2.3
Fusarium	m	m	m	M m	m	m	m
Muskmelon	0.51	0.44	0.78	0.32	0.13	0.19	0.34
Fusarium	m	m	m	(M) m	m	m	m
Aster	0.12	0.38	0.52	0.37	0.57	0.22	0.74
Fusarium	m	m	(M) m	m	m	m	m

*To get the total number of spores per square centimeter, multiply the numbers in these columns by 10^6.

The carbon compounds tested were arabinose, xylose, glucose, galactose, mannose, fructose, maltose, cellobiose, lactose, melibiose, sucrose, raffinose, starch, inulin, dextrin, sodium acetate, glycine, and mannitol.

From the results in Table IV it is clear that certain compounds are more favorable for sporulation than others. The most important feature, however, is the fact that the isolates fall into two distinct groups on the basis of their reaction to the various compounds tested. In the first group are two isolates, navy bean (4) *Fusarium* and aster *Fusarium*. The six other isolates belong in the second group. The isolates in the first group produce more spores per square centimeter on eight of the carbohydrate compounds tested than on glucose. The second group of isolates produce more spores per square centimeter on only two of the carbohydrate compounds tested than on glucose. Mannose and inulin are common to both groups as stimulators of sporulation. This grouping could be carried through into the other three compounds tested, except for the reaction of the navy bean (5) *Fusarium* to sodium acetate. This is the only isolate which gave increased spore production on sodium acetate.

As was the case when different concentrations of glucose were compared, variation of the carbon source affected total spore production and macrospore production differently. For example, navy bean (5) *Fusarium* produced macrospores on only three of the compounds tested, while the muskmelon *Fusarium* produced macrospores on 13 of the compounds tested.

Glucose was not the best compound for the production of macrospores by any of the isolates tested.

(*C*) *The Influence of Certain Nitrogen Compounds on the Sporulation of Eight Fusarial Isolates*

Yeast carbon base (Difco) was the medium employed in these experiments. The nitrogen compound to be tested was added to the medium in sufficient concentration to make 0.5%, and agar to make 2%.

Method of inoculation and the fusarial isolates employed were the same as in the carbon compound assimilation tests.

From the results shown in Table V, it is clear that different isolates possess different potentialities for sporulation on the various compounds tested. For example, pea *Fusarium* produces more spores per square centimeter on five compounds tested than on $NaNO_3$. Only KNO_3 stimulates less spore production than $NaNO_3$. Muskmelon *Fusarium*, however, produced the highest number of spores on $NaNO_3$, while aster *Fusarium* gave maximum sporulation on KNO_3.

No grouping of the isolates is possible with respect to the type of spore produced on the various nitrogen sources.

The Effect of Certain Enzyme Inhibitors on Sporulation of the Muskmelon *Fusarium*

Essentially little or nothing is known about the effect of synthetic compounds on the asexual sporulation of fungi. Horsfall and Rich (13) studied

the effect of a variety of compounds on the sporulation of *Monolinia fructicola* but the fundamental basis for the action of some of these compounds was not known.

It was decided, since a great deal of time had been spent on the study of factors affecting sporulation, that it would be interesting to see whether any light could be shed on more specific factors affecting sporulation. This was attempted by employing a variety of chemical compounds and studying the effect on growth and sporulation.

The test fungus selected was the muskmelon *Fusarium* standard strain. The medium employed was yeast nitrogen base plus 2% agar and the carbohydrate source employed at a concentration of 1%. The various test compounds were added to the agar to make desired molar concentrations, and inoculation was carried out employing a spore suspension of standardized strength. Checks consisted of plates containing the basal medium plus carbohydrate or other carbon compounds, but lacking the test chemical. Results are reported as percentage sporulation and percentage growth of the check cultures.

TABLE VI

THE EFFECT OF SIX CHEMICALS ON GROWTH AND SPORULATION OF MUSKMELON *Fusarium* ON TWO CARBON SOURCES

Chemical	Molar concentration of chemical	Glucose		Sodium acetate	
		% Growth	% Sporulation	% Growth	% Sporulation
Sodium fluoride	10^{-1}	25.8	42	102.1	98.7
	3.3×10^{-2}	39.6	47.3	97.5	96.5
	10^{-2}	46.5	52.1	100	102.1
Iodoacetic acid	10^{-3}	1.6	9.6	99.2	101.3
	3.3×10^{-4}	9.8	36.6	95.7	94.9
	10^{-4}	43.1	40	104.6	92.9
	3.3×10^{-5}	80.1	48.2	91.3	92.12
	10^{-5}	86.7	60	94.9	107.8
Sodium fluoroacetate	4.4×10^{-3}	91.5	37.2	32.3	15.6
	1.3×10^{-3}	90.5	51.6	65.4	51.1
	4.4×10^{-4}	93.7	83.1	89.1	72.3
Sodium salicylate	3.3×10^{-2}	0	0	89.3	90.5
	2.7×10^{-2}	0	0	91.9	97.9
	2.2×10^{-2}	0	0	92.3	99.4
	1.7×10^{-2}	0	0	93.5	101.8
	10^{-2}	43.7	101.2	99.6	94.3
Malonic acid	3.3×10^{-3}	87.3	32.4	—	—
	10^{-3}	91.3	61.3	51.3	42.2
	3.3×10^{-4}	93.3	75.3	101.7	89.4
	10^{-4}	94.1	92.3	102.2	91.3
Sodium arsenite	10^{-3}	99.4	40.9	41.7	32.2
	3.3×10^{-4}	99.7	75.4	88.1	89.2
	10^{-4}	99	85.4	98.6	108.5

(*A*) *The Effect of Sodium Fluoride on Growth and Sporulation, with 1% Glucose and 0.5% Sodium Acetate as the Carbon Sources*

From the results reported in Table VI, it is apparent that, when glucose is the carbon source, sodium fluoride is an effective inhibitor of growth and sporulation, although sporulation is somewhat less sensitive than growth. When sodium acetate is employed as the carbon source, neither growth nor sporulation was affected by sodium fluoride, at the concentrations employed.

(*B*) *The Effect of Iodoacetic Acid on Growth and Sporulation of Muskmelon Fusarium, Growing on two Carbon Sources*

From Table VI it is apparent that iodoacetic acid inhibited both growth and sporulation when the muskmelon *Fusarium* was grown on glucose medium, but was not effective in inhibiting growth or sporulation when sodium acetate was the carbon source.

(*C*) *The Effect of Sodium Bisulphite and Semicarbazide Hydrochloride on Growth and Sporulation of Muskmelon Fusarium Growing on 1% Glucose*

From Table VIII it is apparent that both compounds suppress both growth and sporulation, although sodium sulphite appears less effective at the concentrations employed.

TABLE VII

THE EFFECT OF MALACHITE GREEN ON MUSKMELON *Fusarium* GROWING ON TWO CARBON SOURCES

Concentration of chemical, %	Glucose		Sodium acetate	
	% Growth	% Sporulation	% Growth	% Sporulation
0.001	0	0	—	—
0.0005	2.4	12.4	0	0
0.0001	53	72.3	57.5	17.5
0.00005	68.6	84.4	80.4	21.5
0.00001	80	92.7	105.3	42.7

TABLE VIII

THE EFFECT OF THREE CHEMICALS ON GROWTH AND SPORULATION OF MUSKMELON *Fusarium* ON 1% GLUCOSE

Chemical	Molar concentration of chemical	Glucose	
		% Growth	% Sporulation
Sodium bisulphite	10^{-1}	41.1	32.3
	3.3×10^{-2}	73	57.6
	10^{-2}	83	84.2
	3.3×10^{-3}	91.6	109
Semicarbazide	10^{-1}	1.61	0
	3.3×10^{-2}	10.2	0
	10^{-2}	43.6	37.5
Tetrachloro-*p*-benzoquinone	10^{-3}	0	0
	3.3×10^{-4}	0	0
	10^{-4}	42	61.3
	3.3×10^{-5}	74.2	91.3

(*D*) *The Effect of Sodium Fluoroacetate on Growth and Sporulation of Muskmelon Fusarium, Growing on 1% Glucose and 0.5% Sodium Acetate*

The results shown by Table VI indicate that growth and sporulation are both inhibited by sodium fluoroacetate when sodium acetate is the carbon source. When glucose is the substrate, however, growth is only slightly affected, whereas sporulation is definitely decreased.

(*E*) *The Effect of Sodium Salicylate on Growth and Sporulation of Muskmelon Fusarium Growing on two Carbon Sources*

Study of Table VI shows that sodium salicylate at concentrations above 10^{-2} *M* completely inhibits both growth and sporulation, when glucose is the substrate. At 10^{-2} *M* however, while growth is still sensitive, sporulation is quite normal.

When sodium acetate is the substrate, neither growth nor sporulation is affected to any great extent.

(*F*) *The Effect of Tetrachloro-p-benzoquinone on Growth and Sporulation of Muskmelon Fusarium on 1% Glucose*

The results shown in Table VIII indicate that benzoquinone completely inhibited both growth and sporulation at the highest concentrations, while at the lower concentrations, sporulation was less sensitive than growth.

(*G*) *The Effect of Malachite Green on Growth and Sporulation of Muskmelon Fusarium, on 1% Glucose and 0.5% Sodium Acetate*

The results are shown in Table VII. When glucose is the carbon source, growth is inhibited more by malachite green than is sporulation. When sodium acetate is the carbon source, however, growth is less sensitive to malachite green than is sporulation.

(*H*) *The Effect of Malonic Acid and Sodium Arsenite on Growth and Sporulation of Muskmelon Fusarium on 1% Glucose and 0.5% Sodium Acetate*

The results of this experiment are shown in Table VI. On glucose both chemicals affect sporulation, while growth is only slightly affected. The chemicals inhibit both growth and sporulation when acetate is the substrate.

The potential significance of the results of this section are presented in the discussion.

Preliminary Experiments on the Wilting Principle of Muskmelon *Fusarium*

In view of the evidence adduced that the muskmelon *Fusarium* organism ordinarily establishes itself in the xylem elements of its host (30), it was decided to investigate whether the fungus produced a toxic substance capable of causing the wilt symptoms.

Polysaccharides (33) have been proposed as the cause of wilting in a number of fusarial diseases. These are supposedly produced by the parasite after it has established itself in the vessels of the host, and act as agents of mechanical

plugging. Other mechanical plugging agents have been proposed, such as mycelial growth of the pathogen, tyloses from adjacent cells, or gums produced by either the parasite or the host.

While it is apparent from histological studies of infection that large amounts of mycelium may occur in the vessels of the host it has been shown with the tomato *Fusarium* (34) that this alone is not sufficient to cause wilting. It is also apparent that tyloses are abundant in diseased plants but not in sufficient numbers to cause wilting or aid materially in obstructing the flow of the transpiration stream.

Gaümann (5) and others have proposed that one or more toxic substances are produced and carried in the transpiration stream beyond the area of fungus localization. A compound of this nature, called lycomarasmin, produced by *Fusarium oxysporum* f. *lycopersici* Snyder and Hansen, the fungus which causes tomato wilt, was isolated from 2- to 4-month-old cultures of the organism. Lycomarasmin has not been reported from other forms of *Fusarium*, and the age of the cultures used to obtain this material raises doubt as to whether this compound could be active in infected plants (3), and plants infected with fusarium parasites are generally dead within 2 months.

Fairly recently, Ludwig (20) has demonstrated that, owing to a gum-like substances found in the vessels, only a greatly reduced flow of water passes through the stems of tomato plants infected with tomato wilt *Fusarium*, in comparison with healthy plants.

Recent studies have concentrated also on the role of extracellular enzymes, known to be produced by many *Fusarium* species, in bringing about typical disease symptoms.

Benders' Surprise and Iroquois muskmelon plants, and Peerless watermelon plants, were used in our experiments. They were grown in the greenhouse in light, sandy loam soil, typical of the melon-growing areas of Ontario, with additional fertilizer added to ensure large vigorous plants. When these plants had at least four well-developed true leaves, stems were cut under water under low light intensity and quickly placed in the test solutions.

A virulent strain of the muskmelon *Fusarium* was grown in large flasks containing 250 ml. of nutrient medium and tilted to expose a large area of the solution to air. The standard culture medium consisted of KNO_3, 10 g.; KH_2PO_4, 5 g.; $MgSO_4.7H_2O$, 2.5 g.; $FeCl_3$, 0.02 g.; glucose, 35 g.; water, 1000 ml.

To prepare the test fluid, the fungous mat produced on this medium after growth for 5, 10, 15, and 20 days at 25° C. was filtered off, the mat washed with sterile distilled water, and placed into flasks containing 250 ml. of fresh nutrient medium. After 48 hours, the fungous mats were again filtered off, and the filtrate collected. The mycelial mats were also retained. A second type of test fluid was also employed. For this, a highly concentrated spore suspension of 10^6 spores per ml. was prepared, and 10 ml. of this suspension were added to 240 ml. aliquots of the culture medium in flasks. This gave a

medium containing approximately 40,000 spores per ml. Inoculated flasks were allowed to stand for 48 hours, then the filtrate and the mycelia produced were collected as before. In both cases the media were drip filtered under sterile conditions through four sheets of Whatman No. 1, and three sheets of Whatman No. 3, sterile filter paper. This filtration was repeated three times to insure removal of any fungous spores or mycelium. As a check on this process, portions of the filtrate were plated with V-8 vegetable juice agar, and incubated for 6 days. In one case only following this treatment a single colony of the test organism developed.

In the early tests, the plant cuttings in the test solutions were placed in the greenhouse, but this was abandoned in favor of a room where temperature was held at $25 \pm 1°$ C., and the relative humidity between 60 and 65%. The light source consisted of eight Westinghouse 150-w. reflector lamps. The lights were turned off for 8 hours of every 24-hour period, and the plant cuttings were held under these conditions for a maximum of 96 hours. An electric fan was used to aid air circulation.

On several occasions large, well-developed melon runners were also employed from each of the three types of melons tested. These vines usually had between 24 and 36 leaves, and well-developed stems. Tomato-plant cuttings of the variety Bonny Best were included in several of the tests for purposes of comparison. At least five cuttings of each host plant were employed for each type of filtrate tested, with the ratings for each group being averaged, and further, each type of filtrate was tested on three different occasions.

Dialysis tests were carried out employing dialyzer tubing with a sterilized marble enclosed to act as a stirring agent. Dialysis was carried out against a flow of cold running water, and a rocking device was used to permit a constant agitation of the filtrate by the marble. Chemical tests on the selected filtrates were modified from Harrow (8), and Hawk, Oser, and Summerson (10).

Vascular conductivity of the diseased plants was studied by placing cuttings of control plants, and those which had been exposed to various filtrates, in a water solution of basic fuchsin. The cuttings were allowed to remain in the light in the constant-temperature room for 12 hours, and then selected plants were placed in hot 85% alcohol to remove the chlorophyll. They were then dehydrated through changes of 95 and 100% alcohol, and further cleared in lactophenol.

Wilted plants were rated by the following scale: 0, no wilting; 1, slight wilting; 2, mild wilting; 3, severe wilting; and 4, indicates the plants were dead.

In a preliminary experiment filtrates obtained from 5-, 10-, 15-, and 20-day-old cultures were applied to cuttings of Benders' Surprise muskmelon. The results indicated the probability that some agent was present in the filtrate which caused the plants to wilt.

(A) The Effect of Culture Filtrates on Three Varieties of Melon Plants

In this experiment the filtrates were again obtained from 5-, 10-, 15-, and 20-day-old cultures. The plants employed were Benders' Surprise and

TABLE IX

THE EFFECT OF NON-AUTOCLAVED CULTURE FILTRATES ON THREE VARIETIES OF TEST PLANT

Exposure of cuttings to filtrate, hours	Variety	Rating: Age of the culture from which the filtrate was obtained, days 5	10	15	20	Non-inoculated culture medium A	NA	Tap water A	NA
12	B	1	2	3	3	0	0	0	0
	I	1	2	2	2	0	0	0	0
	P	0	0	1	3	0	0	0	0
24	B	1–2	3	3	4	0	0	0	0
	I	1–2	3	2	4	0	0	0	0
	P	0	1	1	3	0	0	0	0
48	B	2	3–4	4	4	0–1	0	0	0–1
	I	2	3–4	3	4	0	0	0	0
	P	1	1	2	3	0	0	0	0
72	B	2–3	3–4	4	4	0	0	0	0
	I	2–3	3–4	4	4	0	0	0–1	0
	P	1–2	2	2	4	0	0	0	0

NOTE: A = Autoclaved
NA = Non-autoclaved
B = Benders' Surprise
I = Iroquois
P = Peerless

Iroquois muskmelon, and Peerless watermelon. Duplicate sets of plants were employed with one set in autoclaved filtrate, the other in one non-autoclaved. Check plants in autoclaved and non-autoclaved nutrient medium and water were also employed.

The results are summarized in Table IX, but include only non-autoclaved filtrates, since no wilting resulted from any of the autoclaved filtrates.

From these it is evident some substance is present in the filtrates which can cause wilting and that this is produced in cultures only 5 days old in sufficient concentration to cause mild to severe wilting in the test plants exposed to the filtrate for 72 hours. Both the susceptible and resistant muskmelon varieties were affected to about the same degree, while the watermelon variety was less severely affected.

Since no wilting was observed in the autoclaved filtrates, the active compounds were not thermostable. Although most of the check plants were not wilted, in three cases one or two of the five plants in a class were slightly wilted, hence the 0–1 rating recorded for these three cases. This would indicate that the experimental conditions alone were not causing the wilt symptoms.

Also included in this experiment, but not reported on in the table, were much older muskmelon vines and tomato plants. In all cases where the muskmelon vines were employed, wilting of a 2–3 rating was evident after 72 hours in the 5-day filtrate, and of 3–4 rating after 48 hours in the 20-day filtrate. No wilting was observed in the autoclaved filtrates. The tomato plants, however, did not under any conditions exhibit wilting in any way comparable to that shown by the muskmelon plants.

(*B*) *The Effect of Extracts of the 5-, 10-, 15-, and 20-day Mycelial Mats of the Muskmelon Fusarium on Three Varieties of Test Plants*

In this experiment, all the conditions were the same as in the preceding one, except the origin of the test solutions in which the cuttings were placed. The mycelial mats obtained from 5-, 10-, 15-, and 20-day cultures were dried under vacuum at low temperature, and when completely dry, placed under refrigeration for 48 hours, then employed as follows: the dried mats were macerated in a Waring Blendor to which 250 ml. of sterile distilled water had been added. The resulting suspension was then filtered as before, and this extract was used against plant cuttings. From the results it was apparent some factor is present in the cells of the mycelium capable of producing earlier and more severe wilt than the extracellular material obtained from culture filtrates of the fungus.

In the plants wilted either by filtrates or by extracts, browning of the vascular bundles was found, the extensiveness of which depended on the age of the culture from which the solution was derived. The browning produced by the extract of a 5-day-old mat was comparable to the extent of browning found in the plants tested against the 15-day-old culture filtrate.

In histological investigations (30) some evidence was found of large plugs in the vascular bundles as well as fairly abundant mycelia. Normally rooted plants were inoculated by dipping roots in spore suspensions of the organism, and then planting these plants. These plants, when showing appropriate disease symptoms, were cut under water and tested for plugging of the vascular bundles by using a water solution of basic fuchsin. Extensive portions of the vascular system were found to lack the stain.

Similarly plants used in the filtrate experiments were transferred at appropriate times to a solution of basic fuchsin, and these plants were later examined for evidence of vascular obstructions. It was apparent that vascular plugs of some sort were active in restricting the flow of the transpiration stream in excised plants placed in either filtrates or extracts.

(*C*) *The Effect of Filtrates and Mycelial Extracts from 48-hour Cultures on Three Host Varieties*

The culture filtrate was obtained by filtering the nutrient solution in which the spores had been allowed to germinate and grow for 48 hours. The mycelial extract was prepared as before, the young mycelia having been collected by filtration. All other treatments were as before.

The wilting agent previously demonstrated in the 5-, 10-, 15-, and 20-day culture filtrates, and extracts prepared from resuspended mycelial mats of corresponding ages, was produced by the young *Fusarium* cultures. While it was probably not as abundant, judging from the intensity of plant reactions, nonetheless it was present.

Preliminary experiments were carried out, using both filtrates and extracts, in an effort to learn something about the agent or agents inducing vascular browning, wilting, and vascular plugging.

(*D*) *Dialysis of Filtrates*

Filtrates were dialyzed for 24 hours. After 24 hours the 125 ml. of dialyzed filtrate were collected and stored under refrigeration and more filtrate dialyzed until a sufficient amount was collected for testing.

Filtrate collected from 10-day-old cultures dialyzed and tested against Benders' Surprise, Iroquois, and Peerless melon varieties exhibited no loss of wilt potential. The plant cuttings tested against the dialyzed filtrate showed a degree of wilting, vascular browning, and plugging of vascular tissue comparable to that found in the non-dialyzed filtrate. The extract obtained from the resuspended mycelial mats, however, did induce somewhat less wilting as well as a somewhat slower onset of wilt symptoms after dialysis. This seemed to indicate that the extract obtained from mycelial mats contained more than one component active in causing wilt, one of which must be a low molecular weight compound or group of compounds since some passed through the dialyzing membrane.

TABLE X

THE EFFECT OF DIALYSIS ON THE WILTING ACTIVITY OF TWO TYPES OF FILTRATES

Time in filtrates, hours	Variety	Rating			
		Culture filtrate		Filtrate prepared from resuspended mycelial mat	
		Autoclaved filtrate	Non-autoclaved filtrate	Autoclaved filtrate	Non-autoclaved filtrate
24	B	0	2	0	2
	I	0	2	0	2
	P	0	1	0	1
48	B	0	3–4	1	2–3
	I	0	3	1	2
	P	0	2	0	2
72	B	0	4	1–2	3–4
	I	0	3–4	1–2	3
	P	0	2	1	3

It is also apparent from the results in Table X that replacement culture filtrates all lost their activity when heated at 60–65° C. for 10 minutes. With extracts obtained from blended mycelial mats, on the contrary, boiling at 100° C. for 10 minutes did not completely inactivate them.

(*E*) *Determination of the Presence of Lycomarasmin-like Compounds*

An attempt was made to isolate lycomarasmin as a lycomarasmin-like compound from the filtrates of 10-day-old cultures according to the method described by Plattner *et al.* (28). The culture was filtered and the residue discarded. Barium hydroxide was added to the filtrate, and the resulting precipitate discarded. The filtrate was now evaporated to dryness *in vacuo*,

and the residue redissolved in water. Animal charcoal was added, then filtered off. Methyl alcohol was then added and at this point the alcohol should have precipitated the lycomarasmin. No precipitate was formed, indicating that no lycomarasmin was present.

(*F*) *Color Tests of Dialyzed Filtrates and Extracts*

In order to carry out some chemical color reaction tests on the various filtrates (8, 10), 300 ml. of dialyzed filtrates obtained from replacement cultures, blended mycelial mats, and non-dialyzed mycelial mat extract were concentrated to 50 ml. at 50° C. under *vacuo*.

These tests indicate that the dialyzed replacement culture filtrate contained a protein or proteins with a questionable trace of carbohydrate. At least some of the protein complex contained tyrosine, and possibly other amino acids with benzene rings in their structures. This does not prove that the protein active in causing wilt contains these amino acids, only that they were present in the filtrate.

The non-dialyzed and dialyzed mycelial mat extracts both contain a polysaccharide and protein, and the carbohydrate compound appears to be quite abundant.

(*H*) *The Effect of Replacement Culture Filtrates on Plants with Complete Root Systems*

To investigate whether culture filtrates would induce wilting in plants with undamaged root systems, 5-, 10-, 15-, and 20-day-old replacement culture filtrates were prepared as before. Benders' Surprise and Iroquois muskmelon plants were very carefully removed from their pots, and the roots washed free of adhering soil particles under very gently running water. Great care was taken to prevent damage to the root systems.

Filtrates prepared from cultures of four ages, as well as extracts prepared from the resuspended mycelial mats, were poured into 500 ml. Erlenmeyer flasks, 400 ml. of test filtrate per flask. The test plants were then placed in the filtrates with sterile cotton placed at the necks of the flasks to aid in preventing contamination. These plants were placed in the constant-temperature chamber, and allowed to remain there for 72 hours. Light experience was as before.

Both autoclaved and non-autoclaved filtrates were employed. Controls consisted of sterile nutrient medium and sterile tap water.

When autoclaved and non-autoclaved replacement culture filtrates were tested, both the Benders' Surprise and Iroquois melons showed partial to complete wilting. Individual plants did not all react to the same degree, but the same range of reactions were found in both host types tested.

When autoclaved and non-autoclaved mycelial mat extracts were employed, both host plant varieties showed almost complete wilting in all of the plants tested. All control plants remained healthy. When these plants were sectioned freehand, and the sections examined in water mounts, no vascular

browning or vessel plugging was observed. It would seem, then, that this toxic agent acts directly on the root cells in some manner to induce a water deficiency in the plant.

The toxic agent involved in this type of wilting was found to be thermostable at 121° C. for 15 minutes. It is at least partially dialyzable, passes a Seitz filter, and can be demonstrated in the solution outside the dialyzing membrane, but dialysis did not completely remove all the activity even when continued for 24 hours against running water. The toxic agent was stable over a wide pH range.

Discussion

In previous studies in the genus *Fusarium* little attention has been paid to the effect of light on the increase or decrease of spore types in response to light. Harter (9) studied the effect of sunlight and artificial light on the length of three-septate conidia of three species of *Fusarium*, grown on several different culture media, but under otherwise comparable culture conditions. His results showed that conidia were considerably longer in cultures grown in the light than in the dark. His results also indicated that cultures which started their growth in the dark and were then transferred to the light produced macrospores in both the area which originally grew in the dark, and the new areas which developed while under light experience. Also reported was a carry-over effect of light, due to which cultures exposed to light for only 4 days retained their ability to produce macrospores throughout the duration of their growing period when transferred to darkened chambers.

Snyder and Hansen (32) using 10 strains of three species of *Fusaria* stated that no carry-over light effect was present, except when mass or smear inoculation techniques were used. Single spore inoculation techniques did not give a carry-over effect in cultures. Snyder and Hansen, however, used window light and no attempt was made to control temperature and humidity or to standardize the amount of light received by the cultures.

The results presented in this paper indicate that macrospores were produced in response to irradiation by the various fusarium isolates tested. No carry-over of a light effect could be demonstrated in cultures started in the light and transferred to the dark, nor did older cultures grown in the dark produce macrospores on exposure to light, regardless of whether mycelial transfer or single spore inoculation was used in starting the test cultures. Twenty-four- and 48-hour cultures, started by mycelial transfer, which were grown in the dark then transferred to light, did exhibit an apparent ability to produce spores in the older portions of the colonies. This is, however, probably due to new growth response to light in these areas. It has also been reported that microspores are not produced in darkened fusarium cultures. In the present investigation it was apparent that microspores are the only spores produced in the dark, when certain culture media are used. However, V-8 vegetable juice agar stimulates both types of spore production in darkened cultures, though always in greatly reduced numbers.

Temperature has long been known to be a major factor influencing sporulation with many organisms. Similarly it has been shown that with many organisms the combined effect of temperature and light is important. With *Helminthosporium gramineum* it has been shown (14) that the best sporulation occurred under outdoor conditions with 14 to 15 hours of daylight and the average maximum and minimum temperatures of 26.8° C. and 8.2° C. Ramsey and Bailey (29) found that increased ultraviolet irradiation increased macrospore yield of *Fusarium cepae*, but they concluded that increased temperature alone did not do so. From the results of the present investigation it is apparent that either increased temperature alone or increased temperature combined with increased light increased the numbers of macrospores produced in the isolates tested.

It is unlikely that temperature was an influencing factor during these irradiation experiments, since at no time was the temperature at the agar surface found to be more than 1° C. higher than the surrounding air temperature. This was established by means of Petri dishes modified so that the bulb of a thermometer could be placed at the agar surface, but under the Petri dish lid. In a recent paper, Page (25) studying the influence of light on sclerotial production by *Botrytis squamosa* used a system of thermocouples attached to a potentiometer and found the maximum deviation of the temperature at the agar surface from the temperature of the air surrounding the plate to be + 1.25° C. This agrees with the results found during these experiments.

It is interesting to speculate on what the results with the various chemical compounds could mean. All of the chemical compounds used in this study are reported to be inhibitors of enzymes. For example, sodium fluoride inhibits enolase, the enzyme active in converting 2-phospholgyceric acid to phosphopyruvic acid. It must be noted before proceeding further that the inhibitory agents are not actually specific against one enzyme. For example, iodoacetic acid, which will be discussed later, blocks triosphosphate dehydrogenase, an enzyme important in the coupling to the adenylic system, but this is actually only one of many enzymes affected. The sulphydral enzymes are also affected as well as certain krebs-cycle dehydrogenases. The following discussion must then be considered as presenting possible theories rather than conclusive evidence. Other considerations such as accumulation of these agents over the span of the experiments must be remembered.

These experiments employing various inhibitors of glycolysis, fermentation, and aerobic oxidation reactions were undertaken with the knowledge that Nord and his co-workers (21, 22, 23, 24) had for some time maintained that the classical Embden–Myerhof pathway of yeast metabolism was of little or no importance in *Fusarium* species. Recently, however, Cochrane (1) has presented evidence that this pathway not only is present in *Fusarium lini* Bolley, but is operative to quite an extent. Since many of the chemicals used in these experiments are effective against enzymes or substrates operative in this cycle, this lends some support to the ideas to be presented.

The effect of sodium fluoride in inhibiting growth and sporulation when glucose is the substrate indicates that possibly both growth and sporulation

are activated through the Embden–Myerhof pathway. Sporulation, however, is not suppressed as much as growth, indicating, perhaps, that more than one pathway is operative in sporulation. This is borne out even more when one converts percentage total sporulation to percentage spores per square centimeter of colony growth. The number of spores per square centimeter of colony growth rises far above 100%, when compared with the check cultures. Since sodium fluoride does not inhibit growth or sporulation when sodium acetate is the substrate, this may indicate that the latter compound is utilized through some other metabolic pathway.

At this point it should be mentioned that growth of the muskmelon *Fusarium* on 1% glucose and 0.5% sodium acetate after 6 days is comparable with respect to colony diameter and the appearance of the mycelium. Sporulation does not occur as readily on sodium acetate as on glucose, as can be seen from the section on the effect of various carbohydrates on sporulation.

Iodoacetic acid is reported as an inhibitor of both triosphosphate dehydrogenase in the Embden–Myerhof pathway and as a trap for acetaldehyde in fermentation (1, 15, 16). When glucose is the substrate it reduces both growth and sporulation, but again the actual amount of sporulation per square centimeter of colony is increased. It has no effect when acetate is the substrate, and this may lend support to the earlier postulation that acetate may have a different metabolic pathway.

The effect of sodium bisulphite and semicarbazide in inhibiting both growth and sporulation when glucose is the carbon source is interesting. Both these compounds trap acetaldehyde (4, 11); and since pyruvic acid is a precursor of acetaldehyde, this might indicate that if the citric acid cycle is operative in this organism, products produced by the dissimilation of glucose do not enter the citric acid cycle through the oxidative decarboxylation of pyruvic acid. The results may indicate that for growth and sporulation to take place, glucose must be fermented beyond the acetaldehyde stage of aerobic or anaerobic fermentation. This assumption may receive further support from the results with sodium salicylate. Sodium salicylate blocks, among other things, decarboxylase, and could be inhibiting the decarboxylation of pyruvic acid to acetaldehyde. Again, however, acetate may be metabolized through some other system. The effect of tetrachloro-*p*-benzoquinone, which is reported to be an inhibitor of carboxylase in certain species of *Fusaria* (12), adds some support to the tentative conclusion that the glycolysis has to be completed to pyruvic acid which must then be fermented through acetaldehyde for growth to take place. Again sporulation is not nearly as sensitive as growth.

Malachite green, an inhibitor of various dehydrogenase enzymes, blocks both growth and sporulation when glucose is the substrate, but again sporulation is not decreased to the same extent. When sodium acetate is the substrate, sporulation is decreased more than growth.

The results obtained with sodium fluoroacetic acid, an inhibitor of the citric acid cycle through its formation with oxaloacetic acid of a fluoroacetic

acid (26), inhibits both growth and sporulation when acetate is the substrate, but sporulation only when glucose is the substrate. Similar results were obtained with malonic acid, reported as a competitive inhibitor of succinic acid dehydrogenase (18), and sodium arsenite, which blocks the conversion of α-ketoglutaric acid to succinic acid.

These results could be interpreted to mean that energy for growth is obtained from glycolysis and fermentation when glucose is the substrate, while energy for sporulation is obtained through the citric acid cycle. Acetate may be metabolized through various other pathways but finally into the citric acid cycle. On the other hand, this may simply be a difference of susceptibility of two means of growth, "sporulation" and "growth". It is felt, however, that these results do point out interesting avenues for further investigation of sporulation and growth in fungi.

From the results obtained during the wilting principle experiments, it appears that the muskmelon *Fusarium* is capable of producing two, and probably three, fractions in culture active in inducing wilt. One is a protein or proteins and is excreted into the culture medium. The second is a polysaccharide, while the third is a low molecular weight, dialyzable substance which passes a Seitz filter, and is also secreted into the culture medium.

Thomas (33) postulated the production of a wilt-inducing polysaccharide by *Fusarium solani* f. *eumartii* (Carp.) Snyder and Hansen. In his experiments simple culture filtrates were prepared from 15-day-old cultures, and it is possible that much of this polysaccharide, which was released into the culture medium during the 15 days, was due to death and disintegration of some of the fungus cells. This does not mean that materials of this sort might not be active, in part, in inducing wilt, since it has been shown that the muskmelon *Fusarium* establishes and maintains itself in the vessels of its host. With the passage of time, cells of the fungus could senesce and die, and thus release polysaccharides which might become active in mechanical plugging.

Recent work (6, 7, 27, 35) has presented evidence for the role of pectic enzymes in plugging of fusarium-infected crop plants. Since the protein fraction found during the writers' experiments were non-dialyzable and inactivated at 60–65° C., there is good reason to believe it is an enzyme. Muskmelon *Fusarium* was found to utilize pectin as a carbon source in preliminary work, so its ability to produce pectic enzymes is known. It may well be that enzymes of this type are active in inducing wilt in muskmelon plants colonized by the fusarium organism.

Pectic substances occur in most, perhaps all, plant tissues. Pectic enzymes may be operative on the middle lamella or primary cell wall where pectic materials are found. A breakdown of the pectic material of the cell wall could bring about wilting by: (1) large fragments breaking off the cell wall and plugging the xylem elements, thus resulting in a water shortage in the upper part of the plant and thereby producing wilt, or (2) pectic materials breaking down and resulting in a disorganization of the water-translocating system thereby producing wilt (7).

Winstead and Walker (36) found toxic metabolites in culture filtrates which, when applied to plant roots growing in quartz sand, had effects invariably specific for the susceptible varieties of the several hosts. These metabolites were also of low molecular weight and were thought to be concerned in some way with the establishment of the respective fusarium organisms in susceptible varieties.

Culture filtrates and mycelial mat extracts, employed with susceptible and resistant whole plants during this work, produced a toxic agent which induced wilting symptoms in the host plants. This agent was not specific to susceptible varieties only, and there was no apparent difference in the response of the susceptible and resistant varieties. Histological studies have shown that the muskmelon *Fusarium* can invade both resistant and susceptible varieties, and this would be a good correlation, if this toxic agent is active in aiding the organism to colonize its host. It would seem, however, that in nature an extremely high concentration of such a compound would have to be produced in order for it to have any effect. It would also have to be produced in close proximity to the root tissue, and not be adsorbed or bound by soil particles. Many fungus products do not persist in soil, and it is doubtful if a toxic agent of the type mentioned above could persist. It would be interesting to study the role of this type of agent in inducing wilt in plants after the fungus has successfully established itself. This type of toxin might aid the organism in inducing wilt, but since it did not seem to be active in those tests which used plant cuttings, rather than plants with intact root systems, it is difficult to envisage the role such a compound would play in the etiology of wilting. Since its effects on the roots must be on individual cells, one would expect such a low molecular weight compound to be transported in the transpiration stream of cuttings and affect cells in other areas of the plant. The reason for its failure to do so is not at all clear.

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CYTOTAXONOMIC STUDIES ON EREMOPYRUM[1]

PRIYABRATA SARKAR

Abstract

Five species are included in the genus *Eremopyrum*, namely, *E. triticeum*, *E. hirsutum*, *E. buonapartis*, *E. orientale*, and *E. distans*. Of these, the first two are diploids with $2n = 14$, while the rest are tetraploids with $2n = 28$. The karyotypes of the two diploid species are different from those of any other member of the Triticineae in that they have chromosomes with extremely subterminal centromeres. The karyotype of each of the three tetraploid species shows a mixture of two types of chromosomes: seven pairs with extreme subterminal centromeres and the other seven pairs with median or submedian centromeres. This indicates that these are amphidiploids between *triticeum–hirsutum*-like taxa and perhaps some diploid species of crested *Agropyron*. In view of the karyotypic peculiarity, annual habit, and reported sterility barrier, the decision to give *Eremopyrum* generic status is supported.

Introduction

At present most classifications of the Gramineae divide the family into tribes, genera, and species mainly on basis of the structure and arrangement of the inflorescence and its component parts. However, studies on the gross morphology alone cannot provide all the data necessary for an ideal classification. In an attempt to arrive more accurately at a phylogenetic subdivision of the grasses, Avdulov (1) adopted cytological evidence. He showed that the basic chromosome number and chromosome morphology could serve as a foundation not only for the broad divisions of the grasses as a whole but also for the grouping of the genera into tribes and subtribes and of the species in subgenera and sections. His classification is in harmony also with systems based on anatomy and geographical distribution. Recently the method of genome analysis has come to the fore and has been found to be an excellent tool for determining the genetic relationships between taxa.

Being of great economic importance, members of the tribe Hordeeae have been intensively studied by various workers. Among the genera of this tribe, genome analysis has been carried out in detail in *Aegilops* and *Triticum* (7, 10, 11). Before the advent of the genome analytical methods, karyomorphological studies had been made in attempts to provide bases for the classification of the species within a genus. One such study, made on a very extensive scale, is the work of Senjaninova-Korczagina (21) on *Aegilops*. She classified 23 species of *Aegilops* into different sections on the basis of comparative karyomorphology. Kihara (cf. 10) also made a classification of the species of *Aegilops*, but basing his decisions on genomic relationships rather than on comparison of karyomorphology. It is interesting to note that, though approached from different angles, the two classifications are almost in full accord with each other. This tempts one to visualize a parallelism between genome and karyotype, a hypothesis recently advocated by Oinuma (15). For cytogenetic studies, the

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Contribution from l'Institut Botanique de l'Université de Montréal, Montreal, Que. This work was done while the writer was a Research Associate at the Institute.

existence of such parallelism would be particularly helpful where there are strong individual peculiarities in the karyotype of a taxon. In the present study an attempt is being made to show that chromosome morphology can play an important role in taxonomic clarification of the annual wheat grasses.

Material and Methods

The materials used in the present investigation were obtained in the form of seed samples from different sources as listed in Table I. The identifications are based on the descriptions given by Nevski (14) in the Flora of the USSR. The numbers in parentheses are the present writer's acquisition numbers.

Eremopyrum hirsutum was received in the form of a single spike under the name *E. buonapartis* from Professor Naomi Feinbrun of the Botany Department of the Hebrew University in Jerusalem. It had been collected in Central Anatolia, 104 km. south of Ankara, by H. Birand and M. Zohary. In contrast to the glabrous spike of *E. buonapartis*, this spike was very hirsute and in other characters it fitted with Nevski's description of *E. hirsutum*. Melderis (in 16) regarded this taxon as a hairy variety of *E. buonapartis* and used for it the name var. *sublanuginosum* (Drobov.) Meld. The chromosome number and karyotype, as determined by the present writer, however, show it to be distinct from *E. buonapartis*.

Plants of *Eremopyrum buonapartis* grown from seed sample PI 219962 (ERb 246) proved to be much shorter than those grown from the two other samples. Otherwise all three samples fit quite well with the description of *E. buonapartis*.

Somatic chromosomes were studied in temporary root tip squash preparations made according to Tjio and Levan's oxyquinoline method (25). Meiotic preparations were made according to Belling's iron–acetocarmine method after the spikes had been fixed in a modified Carnoy's solution (absolute alcohol: chloroform:glacial acetic acid, 6:3:1). The slides were made permanent by detaching the cover glass in a jar of 95% alcohol and mounting in euparal.

Drawings of both somatic and meiotic chromosomes were made at oil immersion magnification with the aid of a camera lucida.

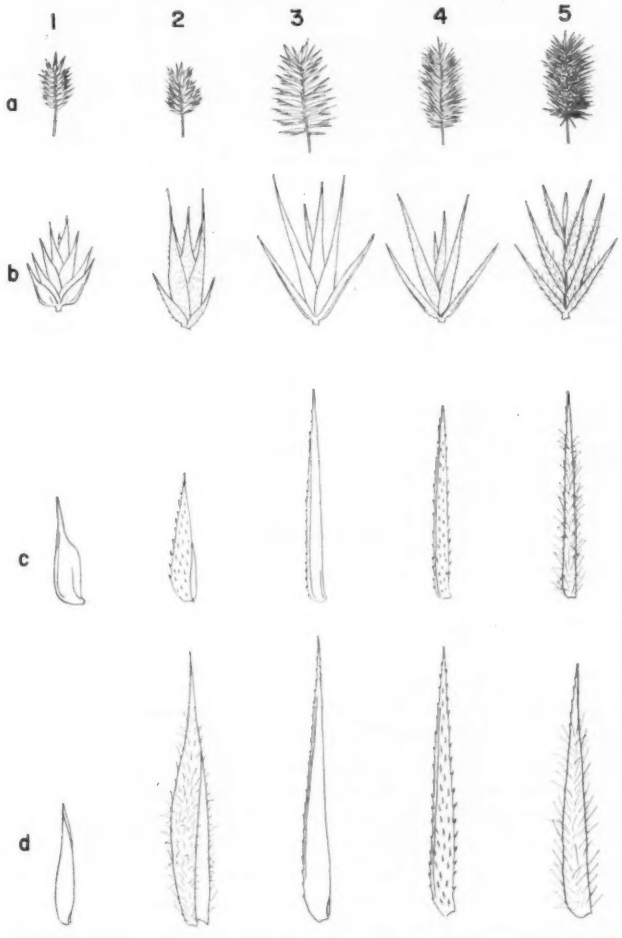
TABLE I

MATERIAL AND ITS ORIGIN

Species	Source	Name and No. under which received
Eremopyrum triticeum (Gaertn.) Nevski (ERt 37)	Dominion Rust Research Laboratory, Winnipeg, Canada	*Agropyron triticeum*, R.L. 5030
Eremopyrum hirsutum (Bertol.) Nevski (ERh 43)	Jerusalem, collected in Central Anatolia	*Eremopyrum buonapartis*
Eremopyrum buonapartis (Spreng.) Nevski (ERb 246, 247, 248)	North Central Regional Plant Introduction Station, Ames, Iowa, USA. Collected in Afghanistan	*Agropyron buonapartis*, PI 219962, PI 219964, PI 219966
Eremopyrum orientale (L.) Jaub. et Spach (ERo 249)	North Central Regional Plant Introduction Station, Ames, Iowa, USA. Collected in Afghanistan	*Agropyron buonapartis*, PI 220568
Eremopyrum distans (C. Koch) Nevski (ERd 39, 245)	North Central Regional Plant Introduction Station, Ames, Iowa, USA. Collected in Afghanistan	*Agropyron orientale*, PI 172693, PI 193264

Morphology and Chromosome Number

Five species are recognized by Nevski (14) under the genus *Eremopyrum*. Morphologically they form a rather homogeneous group. In appearance their closest allies are the crested wheat grasses (*Eu-Agropyron*, *Agropyron* s.str.). However, in addition to the annual habit, they are distinguished from the members of the *Agropyron* s.str. by a series of characters, viz., thick, horny glume; broadened spike-bearing sheath; elliptic-oval or almost round spike;



FIGS. 1–5. Drawings of spikes, spikelets, glumes, and lemmas of the species of *Eremopyrum*. *a*, spikes, × ca. 0.3; *b*, spikelets, × ca. 2; *c*, glumes, × ca. 3.5; *d*, lemmas, × ca. 3.5. FIG. 1. *E. triticeum*. FIG. 2. *E. hirsutum*. FIG. 3. *E. buonapartis*. FIG. 4. *E. orientale*. FIG. 5. *E. distans*.

and brittle rachis. Figures 1–5 show the floral morphology of all the taxa in this group. Some of the easily usable characters in distinguishing the five species, together with their chromosome numbers, are given in Table II. A key for ready identification is given in Table III.

TABLE II

SOME MORPHOLOGICAL CHARACTERS AND CHROMOSOME NUMBERS OF *Eremopyrum*

Species	Culm	Glume	Lemma	2n
E. triticeum	3–50 cm.	0.5–0.7 cm. long, glabrous, shorter than spikelet	Ca. 0.7 cm. long, glabrous	14
E. hirsutum	5–20 cm.	Ca. 0.6 cm. long, hirsute much shorter than spikelet	Ca. 1.4 cm. long, hirsute	14
E. buonapartis	5–50 cm.	Ca. 0.8–1 cm. long, glabrous	Ca. 1.5 cm. long, glabrous	28
E. orientale	5–25 cm.	Ca. 1.1 cm. long, hirsute	Ca. 1.2 cm. long, hirsute	28
E. distans	5–30 cm.	1–1.3 cm. long, villous	1.2–1.3 cm. long, villous	28

TABLE III

KEY FOR IDENTIFICATION OF THE SPECIES OF *Eremopyrum*

Spike glabrous	
Spike small, dense, 1–2.5×0.5–1.5 cm.	*E. triticeum*
Spike larger, less dense, 3–4.5×1.5–2.5 cm.	*E. buonapartis*
Spike hirsute (with short and rough hairs) or villous (with long and soft hairs)	
Glume much shorter than spikelet	*E. hirsutum*
Glume almost equal to spikelet	
Spikelet hirsute	*E. orientale*
Spikelet villous	*E. distans*

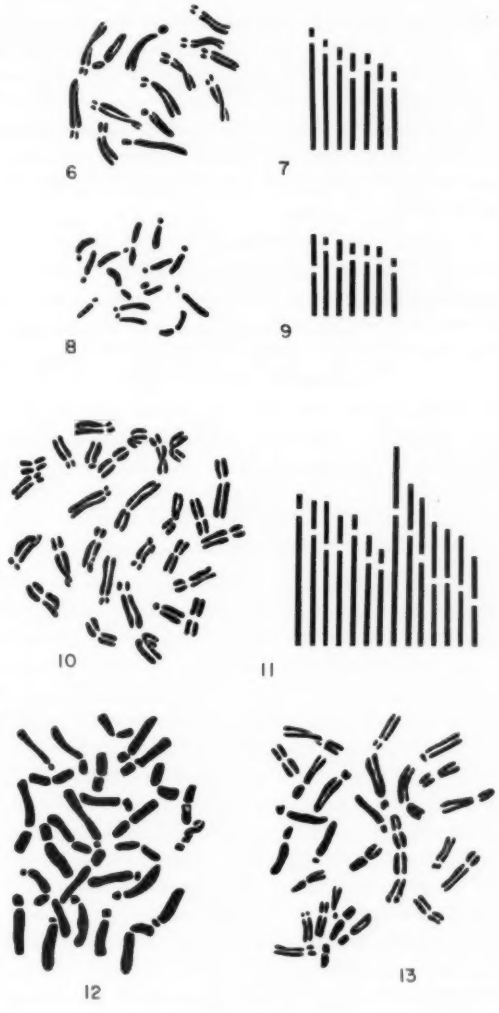
Karyotypes

The karyotypes of all the taxa in this group of annual grasses have been studied and found to be very different, not only from those of crested *Agropyron*, but also from those of all other groups of the Triticineae so far investigated.

The karyotype of *Eremopyrum triticeum* ($2n=14$) can be assumed to represent the basic pattern in this group. Every chromosome in its somatic complement has a subterminal centromere with the shorter arm reduced to a knob-like head (Figs. 6–7). In general, the karyotype of the only other diploid species, *E. hirsutum*, shows only a slight deviation from this pattern, namely, one pair of its chromosome complement (pair 1) has submedian centromere (Figs. 8–9). Further, the difference in length between the longest and the shortest chromosomes is much less in *E. hirsutum* than that in *E. triticeum*.

The rest of the taxa in this group are all tetraploids with $2n=28$. In chromosome morphology they are remarkably similar (Figs. 10–13). The somatic complement of each shows a mixture of two types of chromosomes, with seven pairs resembling those of *E. triticeum*, while the other seven pairs (pairs 8–14) have median or submedian centromeres. This indicates that

each of these taxa is an amphidiploid between two diploid species which contributed the two different groups of chromosomes. One of the putative parents must be a *triticeum–hirsutum*-like species, while the identity of the second parent or parents is a matter of conjecture. Considering the morphological closeness



FIGS. 6–11. Somatic metaphase and idiogram of three species of *Eremopyrum*. FIGS. 6–7. *E. triticeum*. FIGS. 8–9. *E. hirsutum*. FIGS. 10–11. *E. buonapartis*. Magnification × ca. 1100.

FIGS. 12–13. Somatic metaphase of two species of *Eremopyrum*. FIG. 12. *E. distans*. FIG. 13. *E. orientale*. Magnification × ca. 1100.

of the members of *Eremopyrum* to those of crested *Agropyron* and also the chromosome morphology of the latter group (17), it seems likely that the tetraploid species of *Eremopyrum* arose through amphiploidy between diploid species of *Eremopyrum* on the one hand and some diploid crested *Agropyron* on the other. However, the annual habit of the members of *Eremopyrum* poses a serious problem since no diploid crested *Agropyron* is known to be annual.

Meiosis

Meiosis has not been thoroughly worked out in every member of this group. However, a cursory examination shows that meiotic behavior is essentially regular in all the tetraploid taxa. Table IV gives the data for *Eremopyrum buonapartis* and *E. distans*.

TABLE IV

MEIOSIS IN *Eremopyrum*

Species	Number of metaphase-I cells observed with				Total number of cells
	14_{II}	$13_{II}+2_{I}$	$12_{II}+4_{I}$	$12_{II}+1_{III}+1_{I}$	
E. distans (39)	39	—	—	1	39
E. distans (245)	40	2	—	—	42
E. buonapartis (246)	20	—	—	—	20
E. buonapartis (247)	27	—	—	—	27
E. buonapartis (248)	19	—	2	1	22

Discussion

For many years the wheat grasses have been in great taxonomic disorder (cf. 17). Recently some taxonomists (12, 14) have divided the members of the collective genus *Agropyron* into three or four smaller genera, mostly on morphological considerations. On the other hand, genetic connections between species placed in different genera of the tribe Hordeeae prompted some authors (23, 24) to suggest that the phylogenetic relationships between the species of this tribe would be better represented if all were placed in a single large genus. The authors are, however, cautious to add that such a radical step should not be taken until more is known about these relationships.

The reticulate nature of evolution of the different genera of the Hordeeae has been amply demonstrated in recent years (cf. 22). The possibility of gene interchange even at the intergeneric level has naturally taken the edge of the taxonomic delimitations. However, one aspect of the complex interrelationships, particularly in the subtribe Triticineae, must be emphasized. The basic chromosome number of the whole group is seven, and evolution leading to the formation of new taxonomic entities has been mostly by rearrangement or duplication at the genomic level. In other words, different combinations of sets of seven chromosomes have given rise to most of the existing polyploid species through amphiploidy or segmental alloploidy, except in the genus *Agropyron* s.str., where there are a number of autopolyploids (5,

13, 17). Thus the evolution of the taxa in this subtribe has been rather in convergent lines starting from different diploid species. Therefore, an understanding of the interrelationships of the diploid genomes is of major importance in any attempt at a phylogenetic classification of this group of grasses. In fact, Kihara's (6) subdivision of the genus *Aegilops* based on genomic interrelationships is in close conformity with the earlier monographic treatment of the genus by Eig (cf. 10). Moreover, for the genus *Triticum* it has been definitely established that the tetraploid and the hexaploid species are made up of genomes contributed by species of *Aegilops* in addition to the genome from the Einkorn group of wheat (10, 11, 18, 20).

The present writer fully concurs with the view expressed by Stebbins *et al.* (23, 24) that a single large genus would better represent the actual biological situation in this group. However, until such a taxonomic step is taken, it seems desirable to have some consistency in our concept of genus in this complex of taxa. Surely there is as much justification for recognizing *Eremopyrum* as a separate genus as there is for recognizing *Triticum*. It is hoped that the recognition of groups of taxa which are known to be related to distinct diploid species (distinct both cytogenetically and in growth habit) will facilitate rather than hinder further work in this group.

The present karyotypic study confirms the observations by Avdulov (1) as regards *Eremopyrum triticeum* and *E. orientale*. Although he recognized the diversity of the karyotypic pattern within *Agropyron* s.lat., he did not separate *triticeum* and its relatives into a separate group. The distinctive chromosome morphology and the existence of a sterility barrier between *E. triticeum* and other members of the Triticineae (20), however, indicate that it is desirable to give *Eremopyrum* generic status, at least for the present time.

Regarding the possible role of *Eremopyrum triticeum* in the phylogeny of wheat, as postulated by McFadden and Sears (11), it can be said that the karyotypic peculiarity of this species raises great doubts in this respect. This is supported by the fact that no experimental attempts to cross *E. triticeum* with any species of *Triticum* have as yet been successful (20). Recently, Sarkar and Stebbins (18) have suggested from morphological data that the B genome in wheat has been contributed by one or more diploid species similar to *Aegilops speltoides*.

According to Levitsky (9), there is a tendency in the Ranunculaceae for more advanced genera to have karyotypes made up of chromosomes with unequal arms. A similar phenomenon occurs, according to Babcock and his co-workers (2, 3, 4), in the genus *Crepis*. What are considered the more advanced species of *Crepis* were shown to possess asymmetrical chromosomes with a tendency towards a decrease in size and number, as compared with the less advanced species with a larger number of longer chromosomes usually with more or less median centromeres. Further evidence for the possible phylogenetic significance of asymmetrical chromosomes has been furnished by Levan (8) in his studies on *Allium* and by Savchenko (19) in *Vicia*. Avdulov (1) also holds the same opinion about grasses. It thus appears possible

that the genus *Eremopyrum*, with its extremely asymmetrical chromosomes, represents an advanced group in the Triticineae, and the diploid taxa may have been differentiated at an early time.

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CYTOTAXONOMY OF EUPHORBIA ESULA IN CANADA AND ITS HYBRID WITH EUPHORBIA CYPARISSIAS[1]

R. J. MOORE

Abstract

In 14 collections of leafy spurge from Ontario and Saskatchewan the chromosome number is $2n=60$. The problem of the correct botanical name of this plant is reviewed; all the North American collections seen are classed as *E. esula* L. sensu lato. The morphology and cytology of an artificial hybrid, *E. cyparissias* × *E. esula*, is described; its chromosome number is $2n=50$ and meiosis is irregular. The name *E.* × *figertii* Dörfler usually applied to this hybrid is rejected in favor of the prior name *E.* × *pseudo-esula* Schur.

Introduction

Over the past 40 years there has been considerable discussion concerning the correct botanical name of the leafy spurge found in North America. These plants are European introductions and are most commonly known under the name *Euphorbia esula* L. It is disputed, however, whether all or some of the North American population should be referred to the closely related species *E. virgata* W. & K. The European populations appear to be highly variable and some authors question whether the species *E. virgata* should be recognized or whether all variants should be included under the binomial *E. esula*. Both species are native to Europe and western Asia. *Euphorbia esula* is widespread in continental Europe as far south as central Spain, Italy, and the Balkans, extending eastwards through central Russia into Siberia: *E. virgata* occurs in central and eastern Europe south to the northern Balkans and east to Siberia. A number of varieties have been described for both species. Most of these occur in the Asiatic part of the species range. These testify to the great variability of the two species and appear to interconnect them in a complete gradient. Natural hybrids between these two species and *E. lucida* W. & K. have been commonly reported (7) and introgression of genes from these species may be responsible for much of the variation seen in European populations of *E. esula* in recent centuries.

The points in dispute may be stated briefly: are the North American plants identical with the European species *E. esula*, or with *E. virgata*, or are both of these species represented in the New World; and, should *E. virgata* be recognized as a species distinct from *E. esula*, even in Europe? If *E. virgata* is recognized, does the interspecific hybrid reported from Europe occur here and, indeed, should all our plants be regarded as this hybrid?

It is not the purpose of this paper to attempt to resolve all the difficulties of this complex. Especially difficult is the problem of determining the legitimate name of some of the natural interspecific hybrids. The legitimacy of *E. virgata* W. & K. is also disputed by Croizat (3). An extensive survey of the

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populations of the "esula complex", including the culture of living plants and a survey of the chromosome numbers, is needed to understand the morphology and gene exchange of the living plants of Europe and Asia from which our plants are derived.

Although several studies on the taxonomy of the North American leafy spurge have been made, no cytological work has been published. Here, the chromosome numbers of collections from western Canada are reported and the morphology of our plants is discussed. Artificial hybrids between *E. esula* and *E. cyparissias* L. are also described and compared with natural hybrids reported in Europe; it is felt that this may assist in the eventual understanding of the "esula complex" and may be of interest and assistance to those studying the cytology of European collections.

Literature Review

The opinions of recent authors on the correct identity of the North American plants differ widely.

Groh (6) considered that only the species *E. esula* L. was represented among specimens from Canada. Bakke (1) concluded that the European population is highly variable and that it was preferable not to differentiate two species, *esula* and *virgata*. He therefore treated the latter as a synonym of *E. esula*. Morton (9), on the contrary, found no undoubted specimens of *E. esula* in American collections and considered all plants of the midwestern United States to be *E. virgata* and some collections from the east to be *E. virgata* f. *esulifolia* Thell.

In an early paper Croizat (2) expressed the opinion that the North American populations included representatives of four taxa: *E. uralensis* Fisch. ex Link (*E. virgata* W.& K.), *E. pseudolucida* Schur (*E.* × *intercedens* Podpera), *E. lucida* W. & K. var. *androsaemifolia* Koch (*E. virgata* var. *orientalis* Boiss.), and *E. esula* L. At this time Croizat considered that the name *E. virgata* W. & K. was illegitimate and took up *E. uralensis* Fisch. ex Link.

However, Croizat revised his views and in a later paper (3) he discussed the problem at length and concluded that the main bulk of the leafy spurge of North America should be classed as *E.* × *intercedens* Podp. He found only a few specimens of *E. virgata* var. *orientalis* from the New England states. The legitimacy of *E. virgata* W. & K. was defended. Finding that the name *E.* × *intercedens* Podp. was a later homonym of *E.* × *intercedens* Pax, Croizat (4) proposed *E. podperae* Croiz. for that entity. Podpera (10) had originally considered that his *E. intercedens* was a hybrid between *E. esula* and *E. virgata*. Croizat (2,3) used the binomial *E. pseudolucida* Schur as synonymous with *E.* × *intercedens*, presumably applying to an *esula* × *virgata* hybrid. Such is not the interpretation of Hegi (7) who considers *E. pseudolucida* as a natural hybrid between *E. esula* and *E. lucida*. Croizat (3) recognized that *E. pseudolucida* Schur antedated *E.* × *intercedens* Podp. and might be found to be the legitimate name of this taxon. Croizat (3) states that Podpera has used

E. pseudoesula Schur on specimens as probably synonymous with *E.*×*intercedens* Podp. However, Podpera (10) cites *E. virgato-esula* Schur and *E. virgata* var. *latifolia* Schur as prior names of the *esula*×*virgata* hybrid. Hegi (7) also regards *E. virgato-esula* Schur as an *esula*×*virgata* hybrid. It appears therefore probable that *E. virgato-esula* Schur may be found to be the legitimate name of the *esula*×*virgata* hybrid, at present for convenience termed *E.*×*podperae* Croizat (*E.*×*intercedens* Podp.). Croizat (3) regarded the epithet *E.*×*intercedens* Podp. as probably a temporary expedient. An isotype of this entity was at hand for use in North America whereas authentic specimens of the many prior names were not available during the second World War. He considered that the North American leafy spurge matched adequately Podpera's isotype of *E.*×*intercedens*.

Fernald (5) simply reduces *E. virgata* W. & K. to synonymy under *E. esula* L.

In the treatment of the genus *Euphorbia* for the U.S.S.R. by Prokhanov (11) the species with which we are concerned are found in the sections Esulae (11 species, including *E. esula* L.), Lucidae (8 species, including *E. lucida* W. & K.), and Virgatae (13 species, including *E. virgata* W. & K. and *E. cyparissias* L.). The number of species recognized is an indication of the variability to be found among these populations. Several subspecific entities have been raised to specific rank in this treatment. The majority of the species in each of these three sections are Asiatic in distribution. Only to *E. esula* L., *E. cyparissias* L., *E. virgata* W. & K., and *E. subcordata* C.A.M. is an extensive Eurasiatic range attributed.

Materials and Methods

Living plants from Saskatchewan were grown in pots at Ottawa. Most of these plants were selected by the collectors for unusual or extreme morphological characters or for abnormalities in fertility. Some plants, the collector noted, were believed to be sterile (E44, E45) or produced no male flowers (E47) or produced male flowers only in the greenhouse (E48, E50). It was suspected that a fertile–sterile condition correlated with different chromosome number might be found in this species, analogous to that reported in *E. cyparissias* (8).

Root tips were fixed in Randolph's Nawaschin fluid, processed in the usual manner, and stained in crystal violet. Pollen grains were stained in cotton blue and mounted in 45% acetic acid. Grains with a full protoplast were considered to be normal and viable. Specimens were made of the plants grown at Ottawa and they are preserved in the herbarium, Department of Agriculture, Ottawa (DAO). In some cases, as shown in Table I, the collection corresponds to a specimen made before the living plant was sent to Ottawa. This earlier specimen is preserved in the University of Saskatchewan Herbarium and bears the collection number of the University.

Specimens in the herbarium, Department of Agriculture, Ottawa, (DAO), the National Herbarium, Ottawa (CAN), and in the Gray Herbarium of Harvard University (GH) were examined. The latter collections include

TABLE I

CHROMOSOME NUMBERS IN *Euphorbia esula* L.

Accession number	Collector	Date	Location	$2n$
E18	R. T. Coupland, U. Sask. 504	June 18, 1951	Esterhazy, Sask.	60
E19	R. T. Coupland, U. Sask. 505	June 18, 1951	Esterhazy, Sask.	60
E20	R. J. Moore & D. Lindsay	June 26, 1951	L'Orignal, Ont.	60
E32	R. T. Coupland, U. Sask. 507		Mortlach, Sask.	60
E33	R. T. Coupland, U. Sask. 508		Mortlach, Sask.	60
E36	C. Frankton & G. W. Selleck	July, 1953	Sask.	60
E43	G. W. Selleck	1954	Battleford, Sask.	60
E44	G. W. Selleck	1954	Battleford, Sask.	60
E45	G. W. Selleck	1954	Herbert, Sask.	60
E46	G. W. Selleck	1954	Herbert, Sask.	60
E47	G. W. Selleck	1954	Wynard, Sask.	60
E48	G. W. Selleck	1954	Wynard, Sask.	60
E49	G. W. Selleck	1954	Esterhazy, Sask.	60
E50	G. W. Selleck	1954	Esterhazy, Sask.	60

sheets annotated by Croizat. The map of the distribution of leafy spurge in western Canada (Fig. 1) is compiled from specimens in the two Ottawa herbaria.

Cytological Observations

The somatic chromosome number 60 (Fig. 2*a*) was observed in all the collections of leafy spurge listed in Table I. The same number was found also by Pritchard (private communication) in all collections of leafy spurge made in Great Britain. However, Reese (12) has previously reported the number $n=32$, $2n=64$ for *E. esula* L. from Cuxhaven, Germany.

The subsection Esulae Boiss. includes species with the base numbers 8, 9, 10. Theoretically, then, either 60 or 64 would fit the cytological framework of this group. However, *E. cyparissias* L., a closely related species, has the somatic numbers 20 and 40 (Moore and Lindsay (8)), making the base number

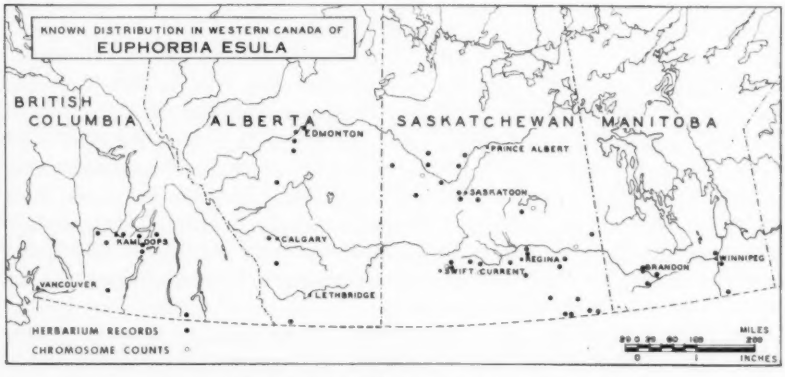

FIG. 1. The distribution of *Euphorbia esula* L. in western Canada.

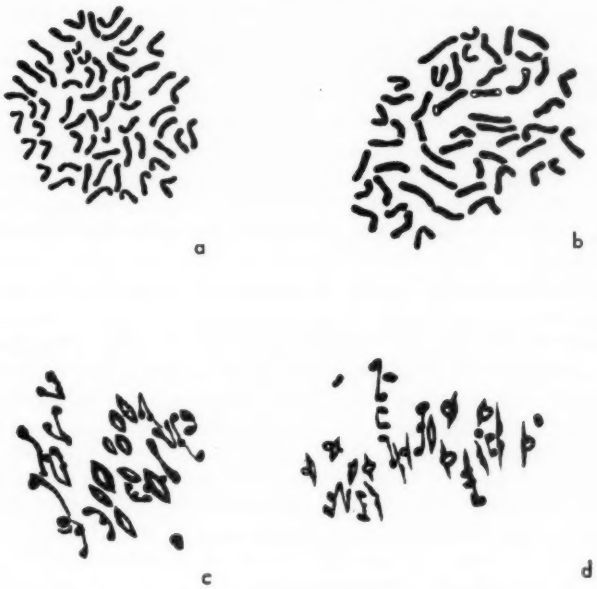

FIG. 2. *a*. Somatic chromosome complement of *E. esula*, ×3000. *b*. Somatic chromosome complement of hybrid, *E. cyparissias*×*E. esula*, ×3000. *c*. I metaphase, plant E4×E18 S1., showing $I^{I} + 17^{II} + 5^{III}$, ×1700. *d*. I metaphase, plant E4×20 S2, showing $4^{I} + 20^{II} + 2^{III}$, ×1700.

10 more probable. The disagreement may, however, reflect a difference between populations of continental Europe and those of areas into which the species has been introduced in more recent times.

Distribution

Leafy spurge has achieved widespread distribution in Canada. It is more abundant in western Canada than in the east and is considered a weed of serious proportions in the west.

In eastern Canada leafy spurge is much less common and occurs in rather localized stands. Records are available from 1 station in Nova Scotia, 3 in Quebec, and 13 in Ontario. In Ontario, it is most frequent in the southwestern portion of the province, roughly in a band extending westerly from the western end of Lake Ontario. In eastern Ontario it occurs rarely about Ottawa and in locations down the Ottawa River toward Montreal.

The distribution of leafy spurge in western Canada is shown in Fig. 1. In addition to the herbarium records indicated on this map, records of infestations in other areas are at hand.

Probably 16,000 acres of the prairie provinces are at present infested. In Alberta and Manitoba the spread of leafy spurge seems to be checked; in

Saskatchewan the problem is more serious. Federal–provincial weed surveys conducted by Dr. R. T. Coupland of the University of Saskatchewan located the weed on 9828 acres on a survey that covered one third of the settled area of Saskatchewan from 1949 to 1955. The infestations are more serious in the southeastern part of the province. About 55% of the infested area is under cultivation, about 20% has been abandoned to leafy spurge, and the remainder is comprised of native plant communities now invaded by leafy spurge. The weed is extending its range rapidly; for example, in one municipality the area infested increased from 342 to 820 acres from 1950 to 1953 (Coupland, private communication).

Little, beyond surmise, is known of the date or method of introduction of leafy spurge into the New World. Groh (6) has mentioned that an early record is available of its occurrence at Newburyport, R.I., in 1870. The occurrence of *E. esula* at Greenstreet, Saskatchewan, is attributed by a local collector to introduction in grass samples obtained in 1912 from Sutton & Sons, Seed Growers, England. This population showed great variation in 1951. The leaves and cyathia are unusually large in specimens collected at this location by H. Groh s.n., June 21, 1935 (DAO). At this time, the population was referred to *E. virgata* by Groh. It seems probable that seed has been introduced and established many times from western Europe in such a manner.

Morphology

In the present study, concepts of the two species have been derived from the type descriptions and photographs. Photographs of the type (Savage Cat. No. 630.62) and of other specimens of *E. esula* in the Linnaean Herbarium and the drawing published with Waldstein and Kitaibel's (16) description of *E. virgata* have been studied. An isotype of *E.* × *podperae* Croizat (*E.* × *intercedens* Podp.) was obtained from the Gray Herbarium, Harvard University. This specimen (Otruba s.n., 1929, Olomouc, Czechoslovakia) is shown in Fig. 3.

Discussions by recent authors indicate the difficulties encountered in separating these species and the characters used for this purpose.

Bakke (1) made a careful study of the size and shape of the glands of North American plants of leafy spurge and found this character to be so variable as to be useless. He made measurements of the leaf width and found that there is "no definite size conformation of a prescribed portion of a leaf". Groh (6) used the relative width of a leaf (the apical half as contrasted with the basal half) and he particularly emphasized the shape of the base of the leaf, the point of attachment to the stem. The leaf of *E. esula* he described as generally broader above the middle, the basal portion narrower and "attenuate to a little petiolate at base". The leaf of *E. virgata* differs in that the basal portion is broader than the apical and forms a "rounded sessile almost clasping base". The texture of *virgata* leaves is also firmer. Similar observations as to the relative width of the apical and basal halves of the leaves have been made by Morton (9), Hegi (7), and Prokhanov (11).

From study of the various descriptions and examination of specimens from North America and Europe it was concluded that the two species should be retained and can best be separated by the following characters.

E. esula L.: Leaves linear-lanceolate or obovate-linear, broader above the middle, tapering to the base, sessile, margins entire or slightly sinuate, leaves thin, not glossy.

E. virgata W. & K.: Leaves lanceolate to ovate, broader below the middle, some widening and almost auriculate at the base, with a short petiole, leaf texture firmer, somewhat stiff, apex acute. The general habit of the plant is more slender, taller, erect, leaves often ascending, leaves on the upper regions of the stem subtending flowering branches often slightly broader than the lower leaves.

There are some differences in the shape of the inflorescence, but this character is variable and is probably influenced by environment and by the age of the plant. This character has therefore been used secondarily to the leaf characters. As Groh (6) pointed out, "the inflorescence appears to be more umbellate in *esula* and thyrsoid in *virgata*." The more thyrsoid appearance is due to the occurrence of fewer rays in the terminal umbel and a larger number of axillary flowering rays rather widely scattered along the upper half of the stem. The present study agrees with the opinion of Groh. Generally *virgata* presents a more slender appearance due to a lesser terminal concentration of flowering rays and their more widespread distribution along the stem. The number of axillary flowering rays is thus usually greater in *virgata* than in *esula*.

Detailed descriptions of the species can be found in the literature, especially Hegi (7) and Prokhanov (11) and need not be repeated here.

E. × *podperae* Croizat (*E. intercedens* Podp.) was considered by Podpera (10) to be an *esula* × *virgata* hybrid. The isotype of Podpera's species (Fig. 3) exhibits characters to be expected in such a hybrid. There is a greater concentration of the flowering rays in the terminal umbel than is usual in *E. virgata*. In typical *virgata* the floriferous portion of the stem is extended and fewer than half the rays are borne terminally. Also the leaves of the isotype are in general linear, not definitely broader at either apex or base—a character intermediate between the species. The firm texture and usually ascending habit of the leaves, however, strongly suggests *E. virgata*. The pollen was found to be only 30% normal. No evidence of the possible fertility of the plant is available, the specimen being in an early stage of flowering.

After careful consideration of evidence available it was concluded that all the Canadian collections seen should be classed as *E. esula* L. sensu lato. Great variation is seen in the populations, in leaf size, shape, and texture and in distribution of the flowering rays. Specimens approaching *E. virgata* in some characters have been seen but none wholly typical of *E. virgata* have yet been seen from Canada. The possibility of the occurrence of *esula* × *virgata* hybrids in the New World cannot be excluded. Presumably Croizat (3) so regarded North American leafy spurge when he classed almost all specimens as

E. × *podperae*. In view of the great natural variability of *E. esula* and our lack of knowledge of the cytology of European populations of the complex it is difficult to agree with Croizat, and, indeed, it seems improbable that an interspecific hybrid, even if partially fertile, would have been commonly introduced into the New World, and more improbable that the species would have repeatedly hybridized here. No collections matching the isotype of *E. podperae* in morphology and pollen condition were found. The approach of some in characters of *E. virgata* can be explained by the high degree of variability well known to occur in *E. esula*. It seems preferable, therefore, to class the variable populations as *E. esula* L. sensu lato and to reserve the name *E. virgata* W. & K. for specimens which conform closely to the type description of the latter species. As will be discussed later, it is considered that *E. virgata* should be retained as a species distinct from *E. esula*. Pritchard (private communication) has arrived at a similar conclusion regarding the leafy spurge of Great Britain. He regards all collections seen to be *E. esula*, in contradistinction to most botanists who consider that both *E. esula* and *E. virgata* occur in Great Britain, the latter species being the more common.

A number of European collections of both species were available for comparison. Among these were specimens typical of *E. virgata*, all from Central Europe and near the type locality of the species (Hungary).

Collections determined as *E. virgata* were the following (DAO): *Austria:* Vienna, Herb. Dr. C. O. Harz. 27.6.1869. *Czechoslovakia:* Somnice v Dubovcich, Bohemia, A. Weideman, 2.6.1886; Eibenschitz, Moravia, Schwöder, June 1880. *Hungary:* Kolosvar, A. Richter, 27.5.1911. *Germany:* Erfurt, W. Rudolph, 27.6.1901?.

The native ranges of the species are given by Hegi (7) as:

E. esula: in Europe, widely distributed from Scotland, Denmark, and S. Sweden, Lake Ladoga in Finland, Novgorod and the Onega Valley on the north, N. Spain, Central Italy, N. Balkans, Roumania, and Central Russia in the south.

E. virgata: Bohemia, Galicia, Poland, Baltic States southeast through the N. Balkans on the south, Central and South Russia on the north, in Siberia as far as Dsungarei and nearer Asia.

It will be seen that *E. virgata* has a more restricted range, occurring in the eastern half of continental Europe, whereas *E. esula* occupies the entire continental mass. Apparently variation becomes more extreme in both species in the eastern part of their range, for several varieties of each species are described from southern Russia and west central Asia. These varieties differ from the type generally in the amount of branching and in the size and texture of the stem leaves. Some varieties are described from limited habitats, such as mountainous or desert regions.

Doubtless, leafy spurge has been introduced into North America many times by seed from different regions of the native range. Different ecotypes, possibly different varieties, may have come to North America. Only in isolated plantings will these populations have remained genetically pure and in these

cases the different environment of the New World may have so altered the phenotype as to make them visibly different from the ancestral stock. The opportunities afforded to various introductions to spread and hybridize may well have given rise to variations not commonly known in Europe. This may have happened especially in western Canada where the spread of leafy spurge has been extensive. For these reasons it was not thought profitable to attempt to classify our plants according to the Eurasiatic subspecific taxa. In time it may happen that distinctive varieties peculiar to definite regions of North America may develop. New gene combinations with possibly partially sterile progeny may be expected now. Some evidence of this has been found in the occurrence of aborted pollen in many North American collections.

Pollen Condition

Examination of pollen samples of North American collections revealed great variation in both size and condition of the pollen. The diameter of full normal grains ranged from 35 to 50μ, most samples falling in the range 45–50μ. The percentage of normal grains varied from 0 to almost 100. In some, but not in all, cases the presence of characters suggestive of *E. virgata* or of *E. lucida* was accompanied by a low amount of normal pollen. These results suggest that some introductions may have carried genes of these related species. It did not seem possible to correlate the varying amounts of defective pollen with any specific morphological characters.

As examples, the specimens of *E. esula* which closely approach *E. virgata* were the following: C. E. Faxon, s.n., June 17, 1878, wasteland, Boston (GH). Pollen 55% normal. This sheet has been annotated as *E. virgata* by Croizat, 1941. D. Kynaston, 12 June 11, 1952, Rock Creek, B.C. (DAO). Pollen 90% normal.

Pollen of 11 European collections of *E. esula* were examined: in nine, the sample was almost 100% normal. The grains followed a size range similar to that of the North American plants.

However, the five European collections cited above as typical *E. virgata* bore grains 30–35μ in diameter, distinctly smaller than the pollen of most American collections and smaller also than European plants classed as *E. esula*. This difference may be indicative of cytologic or genetic characters of value in separating the species.

The isotype of *E. podperae* (GH) bore pollen that was 30% normal, the full grains being 42μ in diameter. This is a condition compatible with its putative hybrid status.

Euphorbia cyparissias × *Euphorbia esula*

During the course of this study crosses were made between *E. esula* and *E. cyparissias*. Seed was obtained from crosses between a tetraploid *E. cyparissias* (No. E4, Moore & Lindsay (8)) and two plants of *E. esula* (Nos. E18, E20 in Table I). Two seedlings were obtained from each cross; three of these (one of E4×E20, two of E4×E18) survived to maturity and have been

grown in field plots for two years. All have proved to be very vigorous and have spread rapidly by underground parts. The flowering period of the hybrid is much longer than that of either species, beginning in early May and continuing until late June. After this date flowering frequently begins again, especially on lateral branches around the periphery of the clump, and continues thus until late September. This later flowering is more sparse. By contrast, *E. cyparissias* flowers here throughout May and *E. esula* flowers from the third week of May to mid-June.

Cytology

The chromosome number of all hybrid seedlings was found to be $2n = 50$ (Fig. 2*b*), as would be expected from the cross of species with somatic numbers of 60 (*esula*) and 40 (*cyparissias*).

Meiosis was studied in each seedling. Because of the small size of the chromosomes and difficulties of fixation, it was possible to analyze meiosis fully in only a small number of pollen mother cells of each plant. Representative I metaphase configurations are seen in Figs. 2*c*, *d*. The average pairing for four cells of each of the two seedlings of parentage E4×E18 was as follows. Seedling 1: $5^{I} + 18.5^{II} + 2^{III} + 0.5^{IV}$; seedling 2: $2.5^{I} + 19.5^{II} + 1.5^{III} + 0.75^{IV}$. Pairing in one cell of plant E4×E20 was $4^{I} + 20^{II} + 2^{III}$. A high degree of homology between the genomes of the two species is indicated by these figures. It is probable that 20 chromosomes of *E. esula* are homoeologous with the haploid complement of *E. cyparissias*. A close relationship between the two species is indicated.

The amounts of apparently normal pollen and the size of the normal grains in parents and hybrids are shown in Table II. Since there was no significant difference in the degree of pairing at meiosis in the two seedlings E4×18, the difference in amounts of functional pollen shown must be attributed to the different genotypes. It is probable that some or all of the parent plants were heterozygous, thus causing a difference in fertility between plants of the same parentage. The size of the pollen grains cannot be taken as an index of the chromosome number in these plants; presumably it is gene controlled. In spite of the occurrence of a considerable amount of visibly normal pollen in two of the hybrids, only a very low degree of fertility has been observed. An occasional capsule is formed as a result of open pollination on the hybrids E4×18 S2 and E4×20, particularly late in the flowering season.

TABLE II

CONDITION OF POLLEN IN PARENTS AND HYBRIDS

Plant number and name	Pollen diameter, μ	% Pollen normal	Chromosome number, $2n$
E4 *cyparissias*	40	90	40
E18 *esula*	42–45	85	60
E20 *esula*	42–45	85	60
E4×E18 hybrid, S1	—	0	50
E4×E18 hybrid, S2	50	30	50
E4×E20 hybrid	40	75	50

PLATE I

FIG. 3. Isotype of *Euphorbia* × *podperae* Croizat.

FIGS. 4 and 5. Hybrids, *Euphorbia cyparissias*×*E. esula*. FIG. 4. Plant E4×E18 S2. FIG. 5. Plant E4×E20 S2.

Morphology of the Hybrids

Two of the hybrids are shown in Figs. 4 and 5. These have a common female parent (*cyparissias*) but a different plant of *esula* as male parent. It is readily apparent that these hybrids are intermediate between the two species. The hybrids are somewhat taller (to 50 cm.) than *E. cyparissias* but are shorter than *E. esula*, as grown here under comparable conditions. The shape of the cauline leaves of the hybrids is generally that of *cyparissias* (narrowly linear) but the size is greater (to 3 mm. width by 35 mm. length) than is found in this species. In the greater leaf size and also in the reduced number of leaves, the hybrids show the influence of the *esula* parent. The occurrence of numerous flowering rays arising in the leaf axils below the terminal umbel, in the upper half of the stem, is a characteristic of *esula*, invariably lacking to this extent in *cyparissias*. In the latter species the flowering rays are concentrated almost entirely in the terminal umbel; only a few axillary rays may be found in the upper third of the stem, immediately below the umbel.

Although the hybrids of different male parentage agree in the above characters, some differences are noticeable between the two, especially when seen in the living condition. The leaves of the hybrid E4×E20 are slightly broader and darker in color than those of E4×E18. The former plant was more delicate and slow growing in the early stages of growth, only one of the two seedlings surviving. A difference in the number and length of the rays in the terminal umbel and also of the lateral rays of the two hybrids is apparent. In this character, length of rays, each hybrid is identical with its *esula* parent.

History and Taxonomy of the Hybrid

The occurrence of natural hybrids between these species in central Europe has been reported by several authors. The hybrid is usually known under the name *E.×figertii* Dörfler (Herb. Norm. 4295, 4296. 1902). There seems, however, to be no valid reason to reject the prior name *E.×pseudo-esula* Schur, published in 1853 (14) and later, with a more complete description, in 1866 (15) and this name is here taken up for the hybrid *E. cyparissias×E. esula*.

Schur (14) reported the occurrence of the hybrid on dry sand along the Zibin River at Hermannstadt (now Sibiu, on Cibinul River, Roumania). He described it as intermediate between the parents. Rechinger (13) reported the hybrid and *E. cyparissias* only at the foot of the Carpathian Mountains near Breitenbrunn (Saxony). Both Schur and Rechinger emphasize that the hybrid is easily distinguished from *E. esula* by the light-green color of the hybrid. Rechinger notes that the pollen of the hybrid is very well developed ("sehr gut entwickelt").

Dörfler (Herbarium Normale, 1902) proposed the name *E.×figertii* for the hybrid and recognized two forms: forma *pseudo-esula* Schur (Herb. Norm. 4296) and forma *polyphylla* Schur (Herb. Norm. 4295). Dörfler considered that the form *pseudo-esula* (*E. pseudo-esula* Schur) closely approached the parent *E. esula* in the size and form of the involucral leaves and is distinguished from this parent by the narrower, smaller, less obtuse but numerous and

thicker stiff leaves of the sterile branches. The form *polyphylla* approaches more closely *E. cyparissias* and is distinguished from this parent by the broader, less numerous leaves of the sterile shoots and branches and by the generally larger involucral leaves and floral parts. Dörfler also notes that the pollen of both forms is well formed ("vollkommen ausgebildet, gut entwickelt"). According to this author the hybrid occurs in lower Austria, in a large area along the Danube from Vienna to the Hungarian border. In some places the hybrid and parents were seen together but in others only the hybrid could be found.

Hegi (7) states that, in addition, the hybrid has been found on the rivers Elbe, Oder (at Breslau, Lüben, Kronen, Küstrin), Rhine (Mannheim), and Danube (Bavaria). The widespread occurrence of the hybrid from eastern Germany, northern Austria, Hungary, and Roumania along the main waterways suggests that the hybrid may have arisen many times and also that pieces of the rhizome may have been spread by means of water.

Isotypes of the two forms of *E.* ×*figertii* Dörfler (DAO) have been examined and compared with the artificial hybrids. The hybrid nature of *E.* ×*figertii* is shown in that the leaves are intermediate in size and number between the parental species and in that flowering rays arise in the leaf axils well below the terminal umbel. The concentration of rays in the terminal umbel is, however, greater than is usual in *E. esula* and in this regard the hybrid approaches *E. cyparissias*. The difference between the two forms appears to the present author to be very slight and scarcely to show the distinctions claimed by Dörfler. Retention of these forms seems to have little practical value.

Pollen of the form *polyphylla* was found to be 36% normal, the full grains measuring 40μ in diameter. Pollen of the specimen of the form *pseudo-esula* was 46% normal in appearance and these grains varied from 40μ to 50μ in diameter. Dörfler's statement that the pollen is well formed seems to imply that the pollen is completely normal, a condition not found in the specimens examined. The size and fertility of the pollen of these specimens from the Herbarium Normale is in general agreement with that found in the artificial hybrids.

It may be concluded that the present artificial hybrids match in all significant characters the isotypes of *E.* ×*figertii* Dörfler.

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